STUDY OF THE MYCETOMES IN INSECTS

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ABSTRACT

The present study consists of two parts. Part-A deals with survey of the mycetomes in different orders of insects. It also includes, the anatomy and histology of the mycetomes of seven species belonging to Anoplura, Coleoptera, Dictyoptera and Hemiptera as well as the histochemical study of the mycetomes of three species (Pyrilla perpusilla Walker, Idiocerus clypealis Leth, Ptyelus sexvittatus Walker). Part-B embodies informations on the isolation and morphology of the mycetomal bacteria, biochemical test, estimation of growth of bacteria in different culture media and their sensitivity pattern to some of the antibacterial drugs.

The paired and tubular mycetomes of Pyrilla perpusilla are attached to the dorsal wall of proctodaeum. Each mycetome consists of mycetocytes of densely granulated cytoplasm and conspicuously large nuclei. The mycetocyte contains granular microorganisms which divide by binary fision.

The paired rod shaped mycetomes of Idiocerus clypealis lie close to the alimentary canal surrounded by fatty tissues. Each mycetocyte contains a single nucleus and embedded with rod shaped microorganisms having single/loose nuclei, [illegible]

The paired trilobed mycetomes of Ptyelus sexvittatus lie entangled in trachae. They are red coloured in male host and light orange in female. Mycetocytes are arranged in columns, with centrally placed nuclei and granular microorganisms.

In Periplaneta americana L the mycetocytes are located singly in fat bodies and are also covered by an enveloping layer. Not necessarily all fat bodies should contain mycetocytes. The mycetocyte has centrally placed nucleus and coloured microorganisms are scattered in its cytoplasm.

In Sitophilus granarius (L.) the mycetocytes are studded in the intestinal ceaca of midgut. Each mycetocyte possesses centrally placed nucleus while the rod shaped microorganisms are scattered in the cytoplasm.

The paired oval shaped and colourless mycetomes of Cimex hemipterus F. are lying on dorsal side of the alimentary canal. Each mycetocyte has a single nucleus. The rod shaped microorganisms are embedded in its cytoplasm.

The mycetomes of Pediculus humanus capitis DeGeer are unpaired and located on stomach disk. These are oval-shaped, coloured yellow and contain a few mycetocytes. Each

mycetocyte has a large nucleus and the granular microorganisms are contained in its cytoplasm.

The mycetocytes of *P. perspicillata*, *P. clypealis* and *P. sexvittatus* are (PAS) positive, thus indicating the presence of glycogen. However, the capability to pick up stain in the three insects differs. Their nuclei and microorganisms become purple red. The mycetocytes fail to take Alcian-blue stain, thereby, suggesting the absence of mucosubstances.

The mycetocytes are Congo-red positive, thus indicating the presence of glyco-protein (amyloids). The intensity to take the Congo-red differs in the three insects (strong in *P. perspicillata*, moderate in *P. clypealis* and weak in *P. sexvittatus*). The nuclei and microorganisms stain brick-red and dark-brown respectively.

The mycetocytes are positive to Millon reaction showing the presence of tyrosine. The microorganisms stain brown in *P. perspicillata*, pale yellow in *P. clypealis* and yellow in *P. sexvittatus*.

The mycetocytes become blue in Bromophenol blue indicating the presence of glyco-protein. The microorganisms are stained blue with this stain. The presence of nucleo-protein (Histone) in the mycetocytes is ruled out because of

negative result with methyl-green-pyronin 'Y' stain.

With Sudan 'B' the mycetocytes of the three insects give positive reaction. Intensity of stain and its distribution differ, thereby indicating the presence of lipids and lipoproteins in different degree.

The mycetomal bacteria isolated from P. perpusilla, I. clypealis, and P. sexvittatus are gram negative, non-sporforming, non-flagelate, non-motile and capsulated. They are capable of growing luxuriantly on nutrient agar, potato agar, blood agar and nutrient broth and can liquify gelatin. On identification these microorganisms come out to be one single species of Klebsiella sp. This identification has been further confirmed by the fact that the bacteria isolated from the mycetomes of these insects have similar morphology and growth in culture media, etc. It is a common symbiont in the mycetomes.

The disc method is used in determining the sensitivity of Klebsiella sp. to a few antibacterial drugs, namely, achromycin, chloromycetin, streptomycin, terramycin, erythromycin, furadantin, penicillin and sulphathiozol. As a result of this test it can be said with confidence that penicillin and streptomycin are equally most effective in arresting the growth of the bacteria. These two antibiotic and erythromycin and chloromycetin (2nd and 3rd) in order of effectiveness), when used in turbidimetric test against Klebsiella shows that chloromycetin come out to be most effective followed by erythromycin.

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P A R T - A

I- INTRODUCTION

The association of microscopic structures of various shapes and sizes located in different parts of the body in certain insects belonging to the order Anoplura, Coleoptera, Diptera and Homoptera have been known since long. Leydig (1850) for the first time observed intracellular organisms in aphids, which have subsequently been named as 'Pseudovitelli', symbiotic organs, green bodies, mycetomes and mysterious bodies (Vaidu, 1945) without much knowledge about their role and significance.

Balbiani (1863) studied the mycetomes of male and female insects and assumed that they were linked with the phenomenon of parthenogenesis. Metchnikoff (1866) however, was of the opinion that the function of the mycetomes in aphids was to supply the food material to the developing embryo. Witlaczil (1882), on the other hand, believed that they were excretory in function; he later abandoned this hypothesis. Portier (1913) concluded that the mycetomes of aphids were an "organs of synthesis", transforming certain substances in the imbibed plant sap into substances utilisable by the insect.

According to Vichanco (1924), Krassilstschik (1887 and 1890) was the first person who thought that mycetomes were symbiotic in nature and contained bacilli. This view has been

supported through the works of Hennequy (1904), Pierantoni (1909) (1910), Buchner (1921), Glaser (1930), Aschner (1932), Paillot (1933), Mansour (1934), Blewett and Fraenkel (1944), Mahdihassan (1947), Steinhaus (1946 and 1949), Koch (1950, 1957 and 1960), and Meyer and Frank (1957), Richards and Brooks (1958), Toth (1959), Gresson and Thread Gold (1960), Buch and Chapman (1961), Buchner (1966), Steinhaus (1967), Lamb and Hinde (1967), Lanham (1968), Hinde (1971) and Than (1972) confirmed the findings of Krassilstschik by using different species of insects.

de Bary (1879) originally used the word 'symbiosis' for association of dissimilar organisms regardless of the end result of such an association, whereas Blochmann (1883), Wheeler (1889) and Gier (1933) used this term only for those unions in which the association was mutually advantageous. Steinhaus (1946) was of the opinion that the term "symbiosis" was a broad one including not only the relationships of mutualism but parasitism and commensalism as well. Obviously, therefore, an association of two dissimilar organisms living together is symbiosis.

According to Mahdihassan (1946 and 1947) males and females of Cicadella viridis differed in their colour and this corresponded with the colour of the mycetomes. Further the different shades of colour in mycetomes and the insect body could be produced in-vitro. Naidu (1945) reported the production of pigment in mycetomes of Ptyelus nebulosus.

The mycetomes, when present, are usually located in the visceral sinus of the abdomen. In some insects these are very small, in others quite large occupying considerable space. The size often varies with the age and development stages of the insect as well as with the sex. Koch (1959) referred the varying size of mycetomes in males and females. In some species the mycetome is a single small body, in others it occurs in the form of pairs or even in groups of small mycetomes. They also vary in colour. They may be highly coloured and easily distinguishable in some, while in others they are white, colourless or transparent and, therefore, cannot be easily distinguished from the surrounding tissues.

According to Beir (1938) mycetomes are of 4 general types;(I) Single, mycetomes harbouring only one symbiote; (II) mycetomes consisting of 2 rather loosely joined organs not enclosed in a common epithelium and harbouring two different symbiotes;(III) mycetomes consisting of two zones, but surrounded by a common epithelium and, therefore, joined into a single organ containing two distinct kinds of symbiotes;and (IV) mycetomes enclosed in a common epithelium harbouring 3 different kinds of symbiotes. The arrangement of symbiotes in the mycetomes is of unlimited nature. Steinhaus (1949) thought that the longer a symbiote lived in the body of the insect species, the more intimate

became the association and more involved became the cymbiotic arrangement. He was also of the opinion that the degree of complexity relating to the histological character of the tissue harbouring the symbiotes and the embryological development, indicated the tenure of the relationship.

Regarding the nature of mycetocytes, Buchner (1953) was of the opinion that they were relatively large cells and highly polyploid. Baudisch (1956) suggested that mycetocytes in the embryo of cockroaches were tetraploid, while in adults polyploid. Baudisch (1958) further suggested that this increase in chromosomal number was due to endomitosis.

Buchner (1955 and 1957) suggested that the mycetocytes undergo mitotic divisions. The division of the cells was controlled by factors such as moulting hormone, pressure of microorganisms within the mycetocytes. Brooks and Richard (1955) and Baudisch (1956) working on Blattella germanica and Periplaneta americana, confirmed the above findings, and pointed out that the mitotic division occured in the later half of the moulting cycle.

A common factor with all the symbiotes was that they were transmitted from generation to generation. Steinhaus (1949) stated that it was only seldom that the reinfection with symbiotes was left to chance. In most of the cases the transmission of symbiotes took place through the opposite host, while in some cases it was also reported to occur through spermatic fluid.

Mansour (1934) working with bettles viz. Rhizopertha dominica, Sinoxylon ceratoniae and Bostrychoplites zickeli, reported the transmission of symbiotes from one generation to the next occurring through testes. According to him the 'Infection' normally first started in outer cells of the testes lobes. The microorganisms from the mycetomes invaded the testes lobes, multipled and mixed with sperms. The symbiotes then passed alongwith the sperm during the copulation into spermatheca of the copulating partner and invaded the descending eggs through their micropyle. Koch (1931) recorded the transmission of microorganism in the females of Oryzaephilus sp., Lyctus sp., Camponotus sp., and Formica fusca occuring through the eggs.

Gier (1936) found that Blochmann bodies of P. americana did not give the Feulgen reaction to any definite degree. Rizki (1954) reported diffuse Feulgen positive reaction in the Blochmann symbionts of B. germanica, a reaction abolished by prior treatment with DNA-ase. Kolb (1959) detected DNA in the mycetomes of Ceratitis capitata and not in Sitophilus granaria and Pediculus sp. Koch (1960), on the other hand, failed to detect DNA in any of the insects studied by him.

Bonhag and Arnold (1961), by using Feulgen and Azure B-bromide stains, found RNA- ase positive neclear material in the mycetocytes of P. americana. They also observed glycogen

in these mycetocytes. In Sitophilus sp., the mycetocytes contained DNA in their nuclei (Musgrave et al. 1962 and Grinyer and Musgrave, 1963). In the symbiotes of aphids Brevecoryne brassicae scattered arrang ment of DNA was observed by Feulgen reaction (Lamb and Hinde, 1966). Further, Louis (1967) observed small amounts of DNA in symbionts, Pseudococcus maritimus after removing RNA- ase by staining w with Pyronin 'Y' and methyl green. The distribution of DNA was scattered throughout the symbionts. He also demonstrated the presence of acid mucopolysacchrides associated wit' proteins in the symbionts of P. maritimus. The occurrence of DNA in the nuclear material of the symbionts from mycetome and mesentera of S. granarius and S. zea-mais (Mots.) was again established by Grinyer and Musgrave 1966, Singh and Musgrave 1966 and Musgrave and Grinyer, 1968.

Although Change and Musgrave (1969) could not demonstrate histochemical presence of DNA in the mycetomesof Pearpsylla, Psylla pyricola; still they maintained DNA bondage with some protein complex which may hinder positive Feulgen reaction. They further observed the presence of proteins by using Hg-bromphenol blue stain. Bhatnagar and Musgrave (1970) furth r estaiblished the occurrence of DNA in the symbionts of S. granarius and they explained that the mycetocytes contained consi erable amounts of DNA occuring as either consolidated dots or spongy material of glycoproteins and mucopolysaccharides.

From a close study of the above investigations concerning the intracellular microorganisms in insects belonging to various orders it appears that though considerable amount of work had been done, yet there are many lacunae which must be studied.

In India Pyrilla perpusilla Walker, takes a heavy toll of sugar cane every year (Rehman, 1941); Idiocerus clypealis Leth, attacks mango blossoms and destroys the entire crop; Ptyelus sexvittatus Walker attacked castor and cotton causing tremendous losses every year; Periplaneta americana L, a house-hold pest feeding on cereal products and other edible, Sitophilus granarius (L.) damages the stored grains; Cimex hemiptorus F., a blood sucking bed-bug causing serious loss of vitality of man and Pediculus humanus capitis Degeer is also a blood sucking louse infesting the human head and capable of transmitting some important human diseases.

From the foregoing it is clear that little is known about the mycetomes and mycetocytes in above mentioned insects. Hence it was considered desirable (i) to study the presence and absence of the mycetomes in the above insects and certain other insects belonging to orders, (ii) to study the anatomy and histology of mycetomes occuring in P. perpusilla, I. clypealis, P. sexvittatus, P. americana, S. granarius, C. hemiptorus and P.h. capitis (iii) to investigate the presence of carbohydrates, protein lipids and lipo-proteins in the mycetocytes of P. perpusilla, I. clypealis and P. sexvittatus by histochemical method in particular.

II- MATERIALS AND TECHNIQUES

In the present investigations Pyrilla perpusilla, Idiocerus clypealis, Ptyelus sexvittatus, Periplaneta americana, Sitophilus granarius, Cimex hemipterus and Pediculus humanus capitis were studied extensively for intracellular microorganisms and mycetomes. These insects were collected from Aligarh and Bulandshahar districts and their surrounding areas.

The insects were killed in ethyl alcohol and dissected in normal saline under binocular microscope for the study of the anatomy of internal viscera and internal relationship with the mycetomes.

The smear technique was adopted to get an idea of the association of mycetomes with the alimentary canal, reproductive organs and fat bodies. This technique proved to be more satisfactory for the study of gross inter-relationship between the mycetomes and internal organs if applied after dissection technique. Giema's Feulgen and Gram stains were used in these studies but the results obtained with Giemsa's proved to be very satisfactory. In Giemsa's stain the slides of smear while moist were flooded with Bouins fluid for 40 minutes. Smear were then washed with 70 per cent 50 per cent alcohols and later on they were washed well with tap water and rinsed with Buffer solution pH 7.0 (Potassium dihydrogen phosphate 1 gm, Sodium hydrogen phosphate 2 g). They were then stained with

Giemsa's stain. A well stained smear presented a beautiful polychrome effect. The microorganisms and their nuclei were of varying shades of pink and red, while the ground cytoplasm was stained mixed blue. In case Gram's stain, the smears were fixed by heat and were stained with aniline gentian violet for 2 to 3 minutes. The extra stain was poured out and then the slides were flooded with Gram's iodine for one minute and finally blotted dry. Aniline xylol was then poured to decolourise the slides till no more colour came out. After washing with water the slides were counter stained with dilute carbol fuchsin for fifteen seconds. Finally the slides were washed in water and allowed to dry. This stain was used for the study of bacteria in order to determine whether the bacteria was Gram's negative or positive.

The Zenker's fixative and Bouin's fluid (aqueous and alcoholic) were tried as fixatives. The specimens were placed in fluid for twelve hours. Excess of the fixative was removed by keeping the material in distilled water for few minutes before taking it through the alcoholic grades (30%, 50%, 70%, 80%, 90%, 95% and absolute alcohols). Some times two washes of the material had to be done in absolute alcohol to ascertain complete dehydration. The dehydrated material was treated with terpinol and methyl benzoate for two hours. The material was transfered to a mixture of methyl benzoate and paraffin (1 party of methyl benzoate and two parts of paraffin)

for an hour. Finally it was transferred to pure paraffin for two changes of two hours each. It was followed by embedding of the material in the paraffin. Serial sections of the material at 5-7 u were cut with the help of rotary microtome. Mayer's albumen was used as a slide adherent. The slides were kept in xylol for about an hour to dissolve the paraffin. Those were later on taken through descending grades of alcohol as prelude to staining them.

Heidenhain's iron haematoxyline and Eosin were used for histological studies. The sections after dewaxing with xylol, were brought to water after passing through descending grades of alcohol. The sections were treated for half an hour to one hour in 2% iron alum solution. They were then rinsed in water and stained with Heidenhan's haematoxyline for one to two hours as in Heidenhain's stain. They were again rinsed with water and treated with iron alum solution for differentiation. The process of differentiation was controlled under the microscope. After satisfactory differentiation sections were washed for an hour in running water. Then they were dehydrated by passing through ascending grades of alcohol and later on transferred to xylol for five to ten minutes. Eosin was used after 90% alcohol during dehydration, whenever counter stain was necessary. Permanent slides were prepared by using canada balsam.

In histochemical studies Bouins, Zenker Helly, Carnoy's and Neutral buffered formaline solution were used as fixatives. The processing of the tissues, dehydration and embedding were

same as outlined above. The following special techniques were employed for the localization of the various histochemical structures on the mycetocytes:-

CARBOHYDRATE

(a) Glycogen:

Periodic acid schiff's technique (PAS) of McManus as given by McManus and Mowry (1960) was employed on material fixed in Bouins and Hellys mixtures. The treated sections after being washed in water, were counter stained with Harris-Haematoxylin for about one minute.

Parallel slides meant for control were incubated for 3-5 hours in saliva at 37 C before treating the sections with the respective stains. Best carmine stain was also employed on Bouins fixed material but the results obtained by this device showed less glycogen than in PAS method. The glycogen stained deeply purplish red.

(b) Acid Mucopolysaccharides (Steedman, 1950).

The slides containing sections were brought to water, stained in a freshly filtered 1.0 per cent solution of Alcian blue in 3 per cent acetic acid for 10 to 30 minutes. The slides, rinsed in distilled water, were placed in 1 per cent Neutral red for 60 seconds and blotted dry. The slides were allowed to dry completely in air and later cleared in xylene and mounted in a synthetic medium. The acid mycopolysacchrides were stained blue green with dark red nuclei.

PROTEINS

(a) Glyco proteins. (Pennhold)

The sections were treated with 1 per cent Congo red for 1 to 6 hours and treated with 1 per cent KI for 60 seconds. They were differentiated in 70 per cent alcohol until only amyloid deposits were stained. The slides were washed with distilled water and stained with Mayers haemalum. 1 per cent acid alcohol, was used for differentiating the slides. The slides were placed in dilute buffer phosphate pH 7.8 and dehydrated, cleared in xylene and mounted in synthetic medium. The amyloid deposits were stained brick red, with blue nuclei.

(b) Millon reaction (Baker modification).

In order to test specific proteins like Tyrosin containing proteins millon reaction test was used. The slides containing sections were placed in a beaker containing reagent and boiled gently for 10 to 15 minutes. The solution was allowed to cool down at room temperature. The slides were washed in distilled water two times and mounted in glycerine jelly. The tyrosine containing proteins were stained red, pink or yellowish red.

(c) General protein.

A modified version of the mercuric bromophenol blue technique of Mazza (1953) was used. The serial sections were deparaffinized in benzene, passed into absolute ethanol and stained in alcoholic Hg-BPB for 15 minutes. After this the sections were passed directly into tertiary butyl alcohol for

one minute followed by two other changes of tertiary butyl alcohol for a total three hours or overnight and finally cleared in xylol and mounted in synthetic mediums. The proteins are stained as deep blue.

(d) Ribonucleic acid (RNA).

The material was fixed in carnoys solution. The slides containing sections were brought to water, stained in methyl green-pyronin Y for 6 minutes. The slides were placed in n-butyl alcohol for 5 minutes, cleared in xylene and mounted in synthetic medium.

Parallel sections meant for control were treated with 10% tricholoroacetic acid (Pearse, 1968) for 15 minutes.

LIPID AND LIPOPROTEIN.

The material was mixed in Bakers calcium cadmium formol for 24 hours at 5°C. The materials were dehydrated in graded alcohols and embedded in the paraffin. The sections were brought to 70 per cent alcohol and stained with Sudan Black 'B' in the 70 per cent alcohol, Pearse (1968). The excess of dye was removed in 70 per cent alcohol, washed in water, and counter stained with 1 per cent aqueous neutral red for 1 minute. The slides were placed in distilled water and finally mounted in glycerine jelly. The lipids stained black or blue or brownish black indicating the presence of lipids or lipo-proteins with red Nuclei.

When parallel section of mycetomes were treated with acetone (for the extraction of lipids and lipo-proteins) and then stained with Sudan Black 'B' in the usual procedure, the granmules in the mycetocytes reacted negatively, showing thereby that they contained lipids and lipoproteins.

										25	26
51.	[illegible]	6...1970	[illegible]		[illegible]						
52.	[illegible]	13...1970	[illegible]		[illegible]						
53.	[illegible]	26...1970	[illegible]		[illegible]						
54.	[illegible]	20...1970	[illegible]		-	-	-	+	-	0	0
55.	[illegible]	30...1970	[illegible]		-	-	-	+	-	0	0
56.	[illegible]	30...1970	[illegible]		-	-	-	+	-	0	0
57.	[illegible]	25.0.1970	[illegible]		-	-	-	+	-	0	0
58.	[illegible]	5.10.1970	[illegible]		-	-	-	+	-	0	0
59.	[illegible]	6.10.1970	[illegible]		-	-	-	+	-	0	0
60.	[illegible]	10.10.1970	[illegible]		[illegible]						
61.	[illegible]	14.10.1970	[illegible]		-	-	-	+	-	0	0
62.	[illegible]	23.10.1970	[illegible]		[illegible]						
63.	[illegible]	26.10.1970	[illegible]		[illegible]						
64.	[illegible]	30.10.1970	[illegible]		[illegible]						
65.	[illegible]	2...1970	[illegible]		-	-	-	+	-	0	0
66.	[illegible]	13.11.1970	[illegible]		-	-	-	+	-	0	0

[illegible] = [illegible]; [illegible] = [illegible]; (+) = observed; (-) = not observed

IV- ANATOMY AND HISTOLOGY OF THE MYCETOMES.

1. Pyrilla perpusilla Walker.

There are two mycetomes which cross each other over the dorsal wall of the intestine (Fig. 1,2 MYT). Individually these look like oblique strands attached to the wall of the proctodaeum. Each mycetome is tubular in shape, brown in colour with the result that it becomes difficult to differentiate it from the alimentary canal. Nevertheless, these do not penetrate the digestive tract. However, it can be easily differentiated from the testes or ovaries.

The smear preparation of mycetome shows large number of red coloured granular cocci which are fairly uniform in size and shape (Fig. 3, GM). Some of them are minute rodlike (Fig. 3, RM). The granular forms of cocci divide by binary fission to produce single granular type microorganisms. The disintegrated cytoplasm takes bluish stain. There are present large number of small, as well as, big vacuoles (Fig. 3, V). These are also filled with granular and rod shaped microorganisms. Some of the granular forms are also seen near the nucleus.

A good number of red coloured giant (Fig. 3, N) and small nuclei are also present. The presence of giant nuclei is a typical pathological condition which may be due to the

invasion of the microorganisms. In order to confirm the nature of intracellular microorganisms the smears were also stained with Feulgen. However, the result with this stain could not be found satisfactory. Further, the microorganisms are found to be Gram negative in reaction.

In the longitudinal section of the abdomen of the host, the paired mycetomes are found surrounded by fine trachae and fat bodies. Each mycetome is externally covered by an outer layer which is called the enveloping layer (Fig.4, EL,E). This layer apparently looks like consisting of three rows of cells-forming columns around the mycetocytes. The cytoplasm is finally granulated. The mycetome consists of the mycetocytes arranged in columns. Each mycetocyte is syncytial in nature and contains a single large sized nucdus surrounded by densely granulated cytoplasm (Fig. 5, 6 MYC). The latter is sometimes vacuolated. In each mycetocyte the nucleus is surrounded by small microorganisms.

2. Idiocerus clypealis Leth.

There is a pair of mycetomes lying in 3rd to 5th abdominal segments of the female mango hopper, (Fig.7, MYT). They are lateroventral in relation to the ovaries which conceal them considerably. Each mycetome is red like in shape and arrested among the fatty tissues. It is coloured pale yellow and varies in length. In the case of male host, the mycetomes are

placed distinctly lateral but very close to the testes and vasa-d frentia (Fig.8, VT). These may be mistaken for testes, but a close study of their structure can help in detecting them from the testes on the basis of size, shape and colour.

Under smear study the mycetome possesses cocci type, granular type and diplococci type microorganisms (Fig. 9,DC)GM). The latter divides by binary fision (Fig. 9, DM). These rod shaped bacilli are also discovered from the vacuoles of the mycetomes (Fig. 9, DM,V). These stained red with Geimsa's stain. The smear contains some scattered nuclei (Fig. 9, N), which confirms the presence of the mycetocytes in the mycetomes. There are also present some granular type of microorganisms which are Gram's negative in reaction.

Histologically the mycetome is syncytial in nature (Fig. 10, SYM). It has a uniform enveloping layer (Fig.11, EL). This layer consists of cells which have been referred as epithetial cells by Mansour (1934) in Rhizopertha dominica F. The probable number of mycetocytes can be presumed on the number of nuclei present in a mycetome. It may be said that the mycetocytes are arranged in three longitudinal layers inside a mycetome. The cytoplasm of mycetocyte is thinly granulated embedded with a prominent nucleus. (Fig. 11, N). The mycetocyte nucleus is suspected to undergo mitotic division. Similarly the occurrence of pycnosis in it cannot be ruled out. The

mycetocyte contains a group of small bacterial cells. Each bacterial cell is uninucleate containing numerous deeply stained rod shaped structures.

3. Ptyelus sexvittatus Walker

There is a pair of mycetomes. These mycetomes are enclosed in mass trachae, lie on either side of the alimentary canal in 4th and 5th abdominal segment of the host. These are spongy in structure and trilobed in form. The mycetomes recorded from female and male hosts vary in size. Their colour also varies-orange in female and red in male. In the female host the mature ovaries occupy a large portion of the abdominal lumen. These conceal the mycetomes which are placed lateral to the alimentary canal entangled in trachae (Fig.12 MYT). The mycetomes in the female host are shorter than those found in the male host. The paired testes are dorsolateral in position extending from fourth to second abdominal segment. These partly overlap the mycetomes which are laterally placed in relation to the alimentary canal (Fig. 13 MYT). The mycetomes maintain their position on account of being entangled in trachae.

Under the smear preparation the mycetome appears to possess granular microorganisms and also a few rod shaped microorganisms (Fig. 14 GM, RM). Its cytoplasm contains

distinct vacuoles (Fig. 14 V). The cytoplasm stained reddish blue with Giemsa stain (Fig. 14 C). This reaction might be due to the secretions of acidic nature from the microorganisms as a result of their activity. This presumption gets support from the findings of Mehdihassan (1967). Binary fision have been recorded in the microorganisms whose dividing nuclei are Gram's negative in reaction.

A study of the longitudinal section of the host abdomen shows that the microorganisms are surrounded with fat bodies. Besides, the mycetomes are externally covered over by a continuous enveloping layer (Fig. 15 EL). These mycetocytes are seen at a periphery just beneath the enveloping layer. Each mycetocyte has a single nucleus placed in its centre (Fig. 16 SYM). The nuclei are gigantic in size and surrounded by small granular microorganisms. The cell wall of the mycetocyte has granulated cytoplasm. The phenomenon of pycnosis is common in the mycetocytes. This may be taken as a sign of distribution of the cell. Such disintegration may be attributed to the activity of the microorganisms.

4. Periplaneta americana (L).

The body cavity in both sexes of P. americana (L) is filled with globlated fat bodies entangled in tracheae. Some of the fat cells contain one mycetocyte in its centre (Fig. 17, MYC). The mycetocytes are so arranged in fat cells as to give the

impression of the presence in rows. These are surrounded by another inclusion of the fat cells. The mycetocyte is cellular with distinct cell membrance. Its cytoplasm contains conspicuous visible symbionts.

The smear preparation of the fat body shows that besides the mycetocytes there are large number of granular shaped microorganisms and a few rod shaped microorganisms (Fig. 18, GM, RM). The smear also contains some cytoplasmic vaculated speces, filled with the granular type microorganisms (Fig.18, C,V). Both the types of microorganisms are stained reddish blue with Geimsa's stain.

A microscopic study of longitudinal section of fatty tissue of the P. americana show the presence of mycetocytes. Each mycetocyte is easily distinguished from the fatty tissue by an enveloping layer (Fig. 19, EL). It contains granular shaped microorganisms. The nucleus is large and centrally placed (Fig. 19, N). The mycetocytes also contain cytoplasmic vaculated spaces.

6. Sitophilus granarius (L).

In Sitophilus granarius (L) the mid-gut contains several intestinal caeca* like projections. The mycetomes are lodged inside the caeca as assemble of cells and white in colour. Each mycetome contains a few mycetocytes and a

* In Calandra oryzae the word caeca is used by Murry and Tiegs (1934).

distinct enveloping membrane. In the larvae of both, the mycetomes are 'U' shaped. In the pupal stage as a result of metamorphosis the mycetomes break down and then individual mycetocytes so liberated enter into the intestinal caeca of the mid-gut (Fig. 20, MYC).

Under the smear preparation of intestinal caeca containing mycetocytes, it is observed that a large number of rod shaped microorganisms are present in the mycetocytes (Fig. 21, RM). These microorganisms divide by binary fision (Fig. 21, DM). The cytoplasm of the mycetocytes contains distinct spaces of varying dimensions and stains blue with Geimsa stain. The nucleus is stained red with the same stain (Fig. 21, N).

The longitudinal section of intestinal caeca shows that it contains mycetocytes, enveloped by the covering membrane of the mycetome (Fig. 22, MYC, EL). The mycetocytes are placed in association with the epithilium of the intestinal caeca. Each mycetocyte has a faint cell wall. It has a single nucleus which is centrally placed and gigantic in size (Fig. 22, N). The rod shaped microorganisms are present around its nucleus.

6. Cimex hemipterus F.

The mycetome of Cimex hemipterus (bed bug) are paired, lying on either side of the alimentary canal from third to

fourth abdominal segments (Fig. 23 MYT). They are oval or pear-shaped and white in colour. The mycetomes are suspended through delicate thread from the base portion of the testes. They are entangled in tracheae coming out from the fourth abdominal spiracle.

In the smear preparation of the mycetomes, the rod shaped microorganisms are present (Fig. 24, GM, RM). These microorganisms stained red with Geimsa stains. The granular form microorganisms are also seen. Its cytoplasm contains distinct vaculated spaces and reddish blue with Geimsa stains (Fig. 23, C,V).

Histologically the mycetome is easily distinguished from the fatty tissues. It has a continous enveloping layer and the mycetocytes are arranged in longitudinal columes (Fig.25, MYC, EL). Each mycetocyte has single nucleus, which is centrally placed, gigantic in size and divides by binary fission (Fig. 25, N). Around the nucleus small granular shaped microorganisms are present. The phenomenon of pycnosis is common in the mycetocytes.

7. Pediculus humanus capitis De Geer.

The mycetome in the Pediculus humanus Capitis is located in small organ known as the stomach disk (Aschner 1934). The

* Studied in the male bed bug only.

mycetome is minute, oval shaped and attached to the outer ventral mid-gut (Fig. 26, MYT). It lies exactly in the mid ventral line of the body. The mycetome is yellow in colour. It can easily be recognised against the red back ground of the blood contained in the stomach.

The smear preparation of the mycetome show that large number of granular shaped microorganisms are present (Fig. 27, GM). The mycetome contains cytoplasmic vaculated spaces, filled with granular shaped microorganisms (Fig. 27, C). With Geimsa stain the granular shaped microorganisms are stained red. The smear contained some scattered nuclei (Fig. 27, N).

A study of the longitudinal section of the host abdomen shows that the mycetome is covered over by a continous enveloping layer (Fig, 28, BL). The mycetocytes are seen just beneath the enveloping layer. Each mycetocyte has a large sized centrally placed nucleus (Fig. 28, MYC, N). Around these nuclei some granular shaped microorganisms are present. The cytoplasm is vaculated.

Fig.1. Abdomen of female Pyrilla perpusilla Walker, dissected to show the anatomy of internal viscera and their internal relationship with mycetomes.

CP - Crop; COD - Common oviduct; MI- Mid-intestine; MT - Malpighian tubules; MYT - Mycetomes; OD - Ovlduct; OE - Oesophagus; OV - Ovary; RM - Rectum and SPT - Spermatheca.

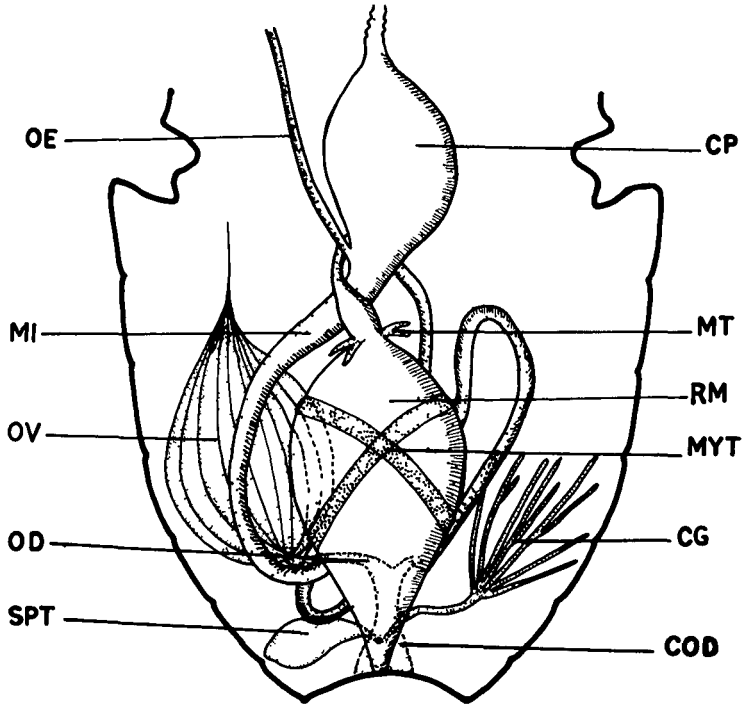


FIG.I

Fig.2. Abdomen of male adult of P. perpusilla, dissected to show the anatomy of internal viscera and their internal relationship with mycetomes.

AG - Accessory gland; CP - Crop;
EJD - Ejaculatory duct; MI - Mid intestine;
MT - Malpighian tubules; MYT - Mycetomes;
R[illegible] - Rectum; T - Testes and VD - Vas deferens.

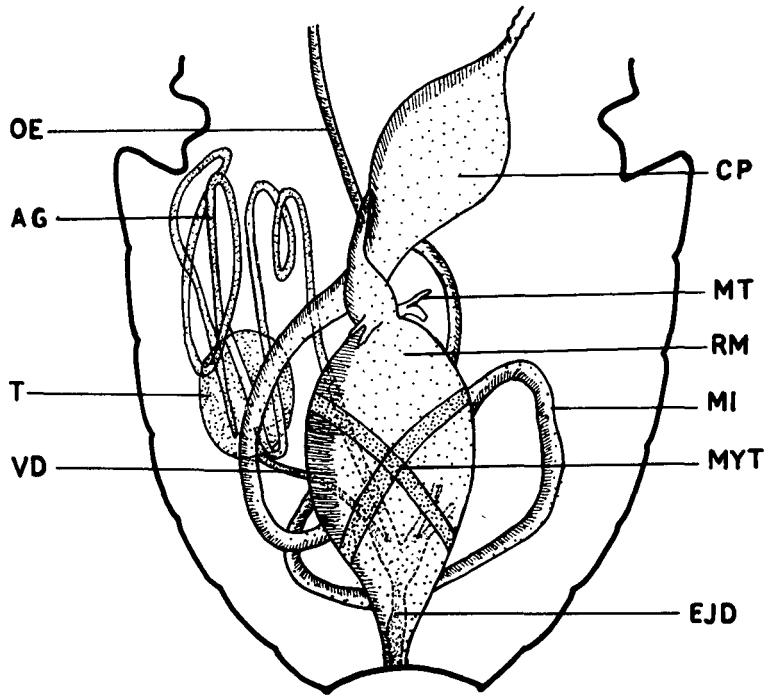


FIG. 2

Fig.3. Photomicrograph of a portion of the smear of mycetomes of P. perpusilla showing microorganisms and nuclei, stained with Giemsa stain.

C - Cytoplasm; DM - Dividing microorganisms;
GM - Granular microorganisms; N - Nucleus;
RM - Rode like microorganisms and V - Vacuole.

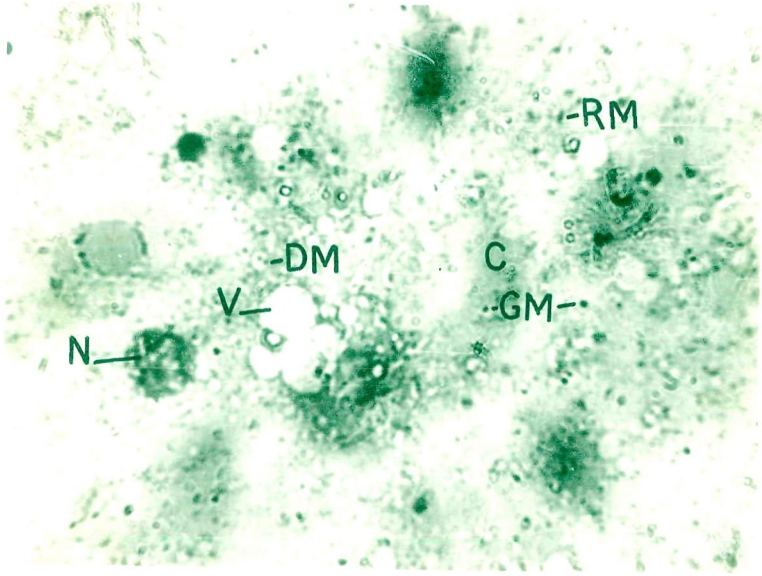

-RM
-DM
C
V
GM
N

Fig.4. Phtomicrograph of transverse section of mycetome of P. perpusilla showing the division of nucleus of the mycetocytes.

EL - Enveloping layer; MYC - Mycetocytes and N - Nucleus.

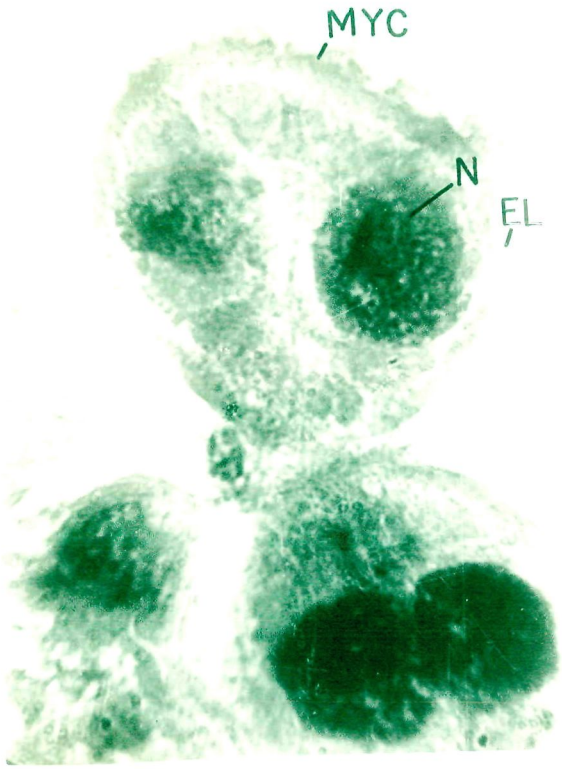


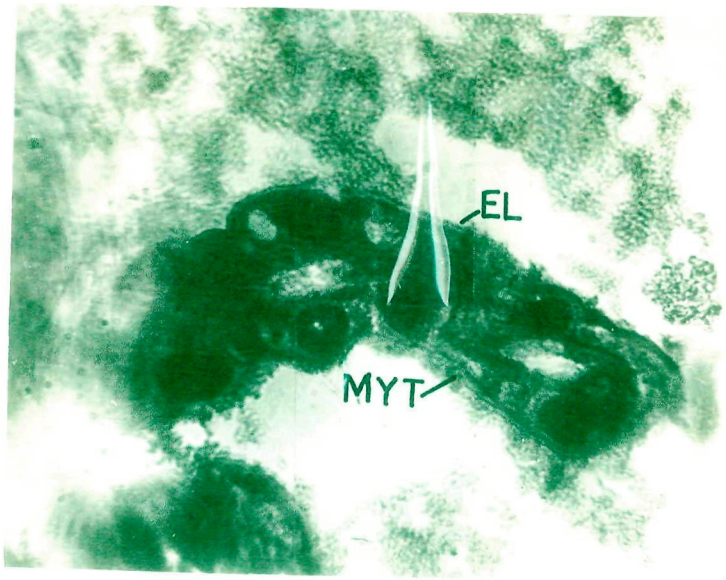
FIG.4

Fig.5. Photomicrograph of a longitudinal section of mycetome of P. perpusilla showing disintegration of mycetocytes.

EL - Enveloping layer; MYT - Mycetome; MYC- Mycetocytes and N-nucleus.

Fig.6. Photomicrograph of a transverse section of mycetome of P. perpusilla showing disintegration of the mycetocytes.

EL - Enveloping layer; MYC - Mycetocytes and N - Nucleus.


EL
MYT


MYC
N
EL

Fig.7. Abdomen of adult female of Idiocerus clypealis Leth., dissected to show the anatomy of internal viscera and their internal relationship with mycetomes.

COD - Common oviduct; MI - Mid-intestine;
MYT - Mycetome; OD - Oviduct; OV - Ovary;
RM - Rectum and SPT - Spermatheca.

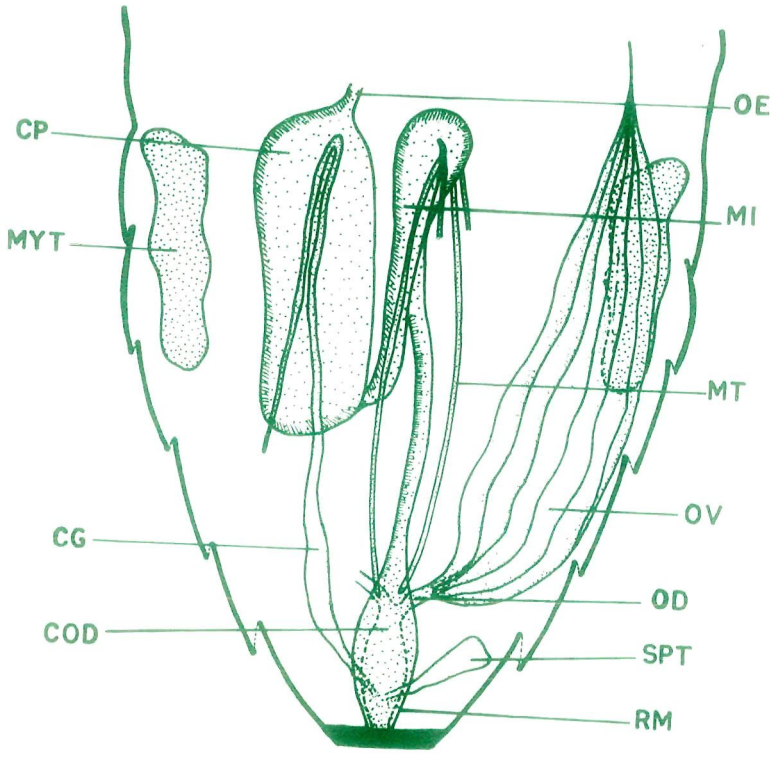


FIG. 7

Fig.8. Abdomen of adult male of *I. clypealis* dissected to show the anatomy of internal viscera and their internal relationship with mycetomes.

AG - Accessory gland; CP - Crop; EJD - Ejaculatory duct
MI - Mid-intestine; MT - Malpighian tubules;
MYT - Mycetome; OE - Oesophagus; RM - Rectum;
SV - Seminal vesicle; T - Testes and VD-Vas-deferens.

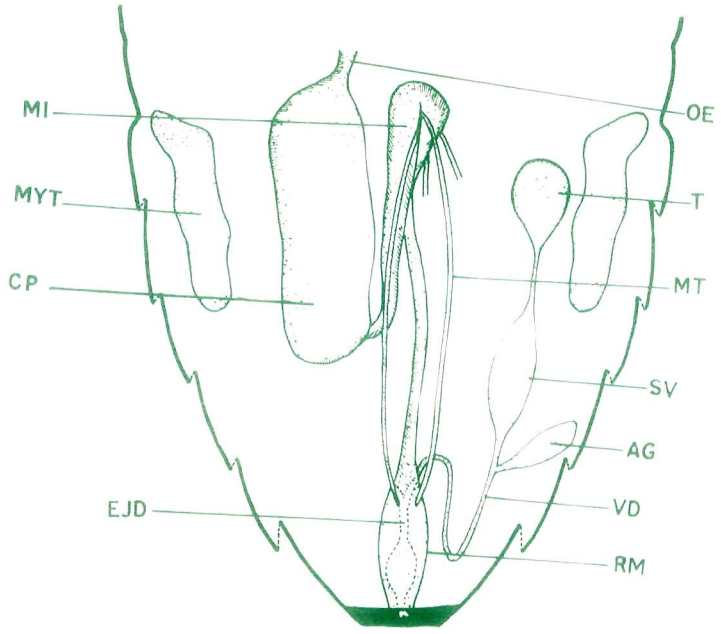


FIG. 8

Fig.9. Photomicrograph of a portion of the smear of mycetome of I. clypealis showing microorganisms and nuclei stained with Giemsa stain.

C - Cytoplasm; DM - Dividing microorganisms;
RM- Rod like microorganisms; N - Nucleus; and
V - Vacuole.

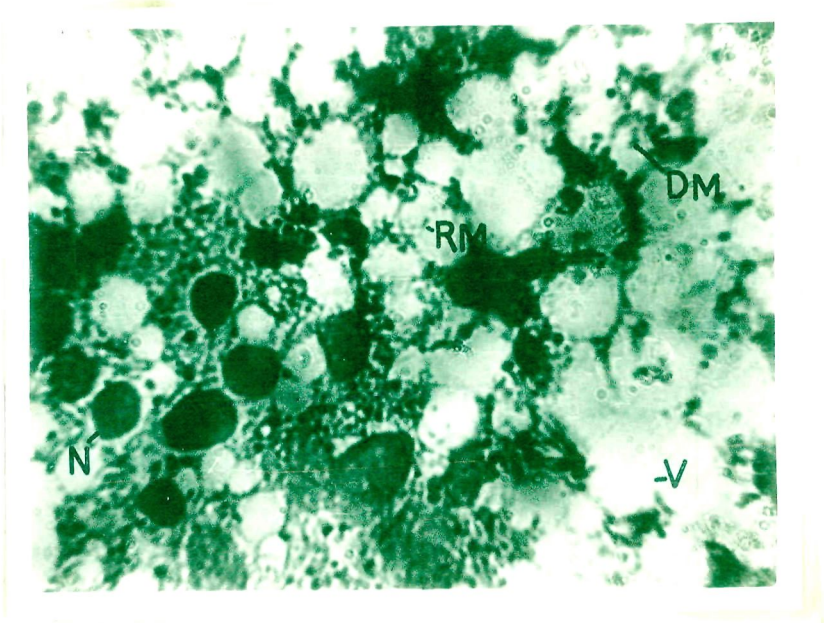


FIG. 9

Fig.10. Photomicrograph of a longitudinal section of mycetome I. clypealis showing disintegration of the mycetocytes.

EL - Enveloping layer; MYT - Mycetome; and TRC- Tracheal cut end.

Fig.11. Photomicrograph of a longitudinal section of mycetome of I. clypealis showing syncytium.

MYC - Mycetocyte; N - Nucleus and SYN-Syncytium.

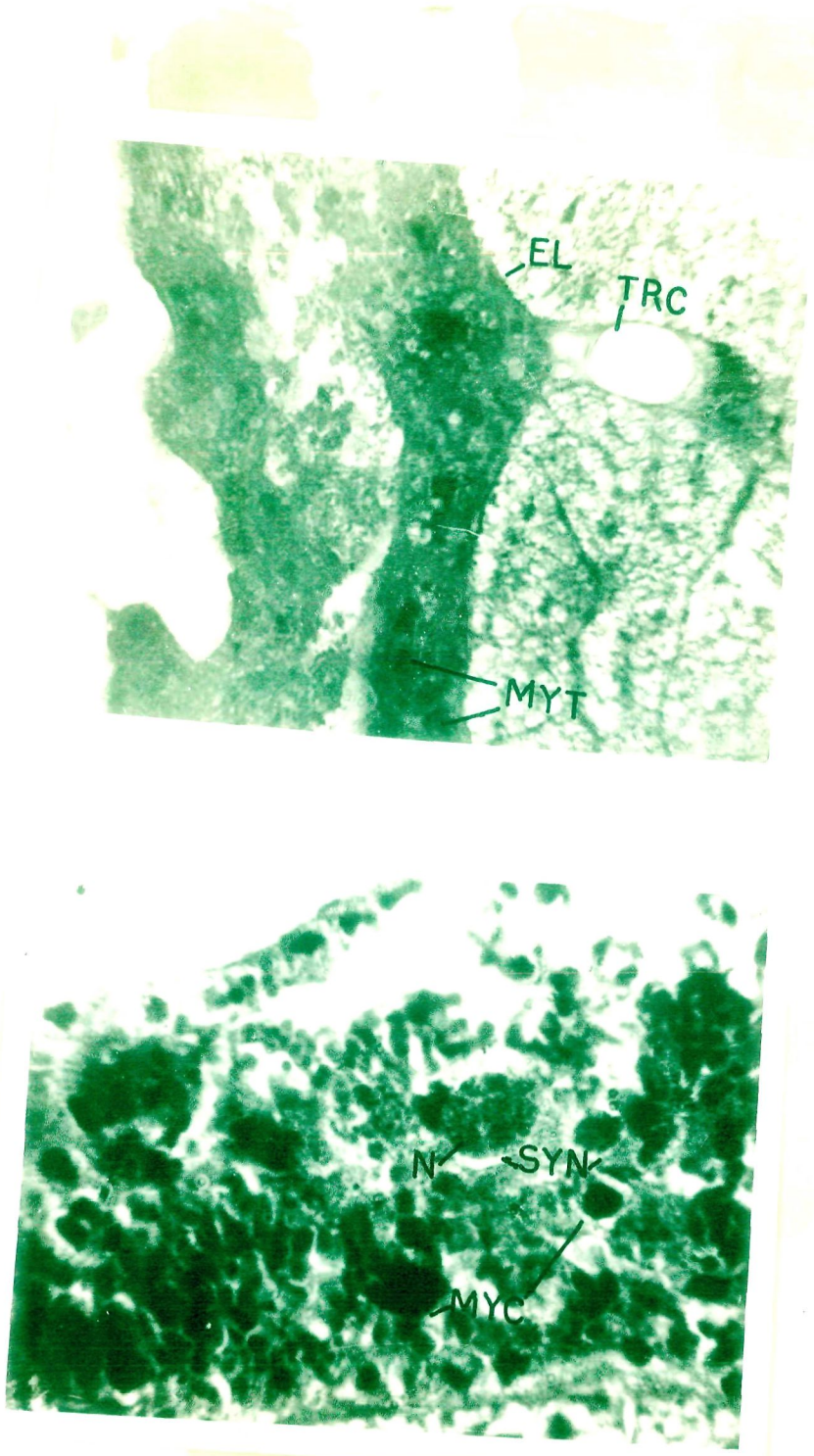


FIG. II

Fig.12. Abdomen of adult female of Ptyelus sexvittatus (Walker), dissected to show the anatomy of internal viscera and their internal relationship with mycetomes.

COD - Common oviduct; MI - Mid-intestine; MYT - Mycetome; OD - Oviduct; OV - Ovary; RM - Rectum and SPT - Spermatheca.

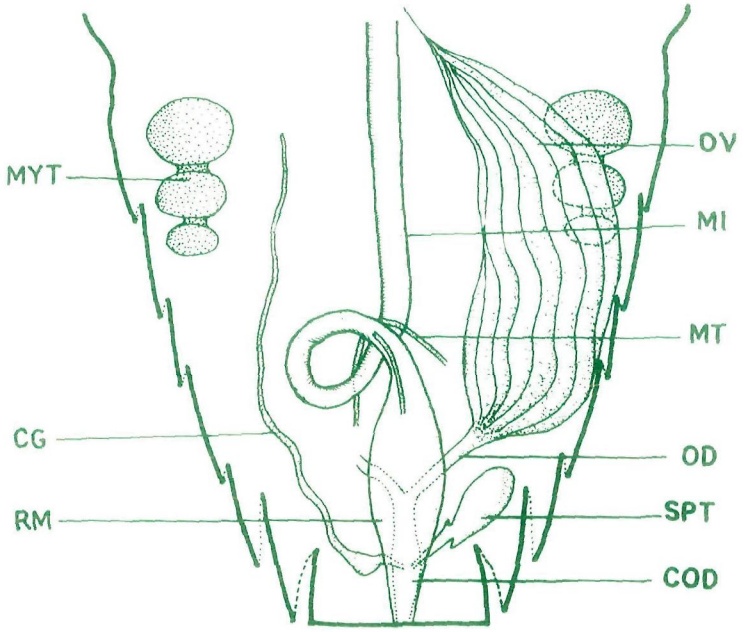


FIG. 12

Fig.13. Abdomen of adult male of P. sexvittatus dissected to show the anatomy of internal viscera and their internal relationship with mycetomes.

AG - Accessory gland; CP- Crop; EJD - Ejaculatory duct; MI - Mid-intestine; MT - Malpighian tubules; MYT - Mycetome; OE - Oesophagus; RM - Rectum; SV - Seminal vesicle; T - Testes and VD - Vas-deferens.

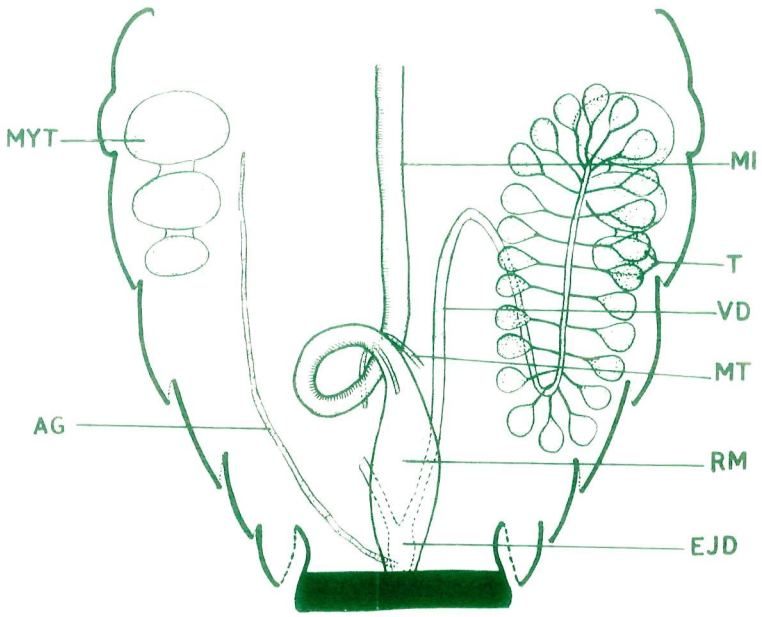


FIG.13

Fig.14. Photomicrograph of a portion of the smear of mycetome of D. sexvittatus showing microorganisms and nuclei; stained with Giemsa stain.

C - Cytoplasm; DM - Dividing microorganisms; GM- Granular microorganisms; N - Nucleus and V - Vacuole.

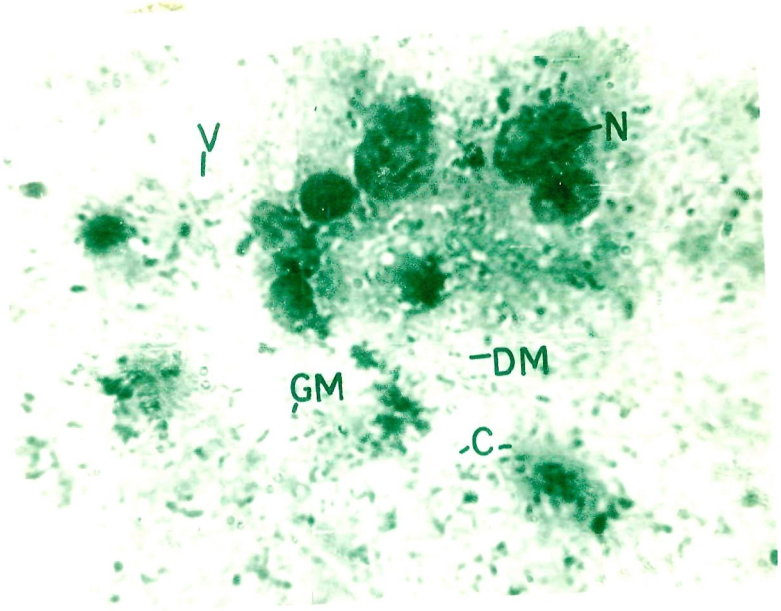


FIG. 14

Fig.15. Photomicrograph of a longitudinal section of mycetome of P. sexvittatus showing disintegration of the mycetocytes.

EL - Enveloping layer; MYC - Mycetocytes; and
N - Nucleus.

Fig.16. Photomicrograph of a portion of mycetome of P. sexvittatus showing the detail of the outer enveloping layer of mycetocytes.

EL - Enveloping layer; MYC - Mycetocytes; and
N - Nucleus.

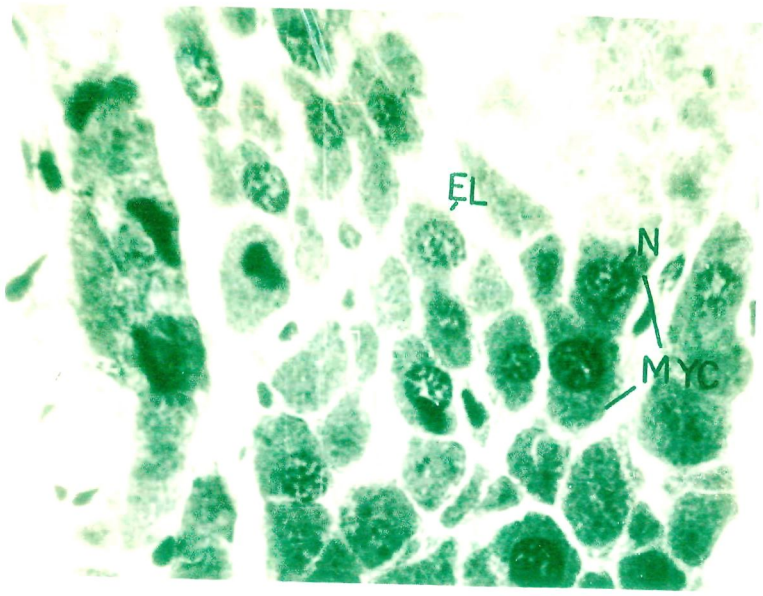


FIG. 15

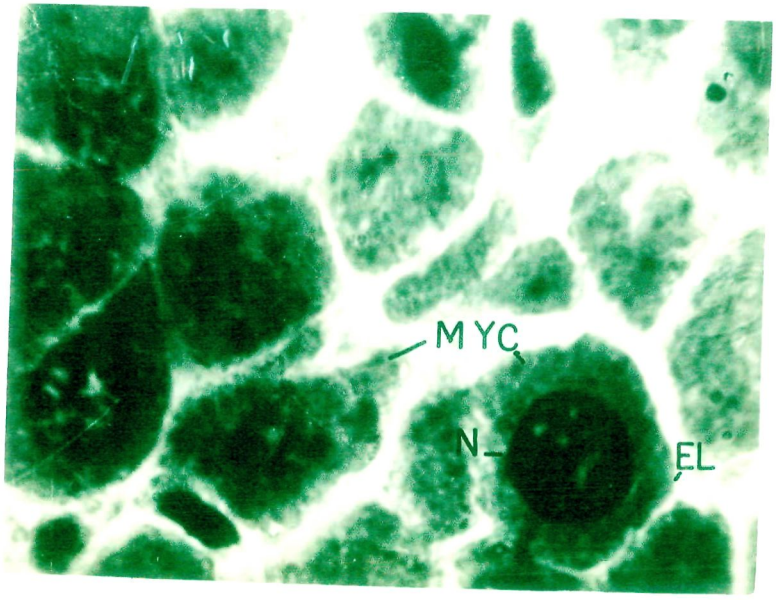


FIG. 16

Fig.17. Abdomen of adult of Periplaneta americana L., dissected to show the anatomy of intestinal viscera and their relationship with fat bodies containing mycetocytes.

C - Cytoplasm; H - Heart; GM - Granular microorganisms; MYC - Mycetocyte; N - Nucleus and T - Trachea.

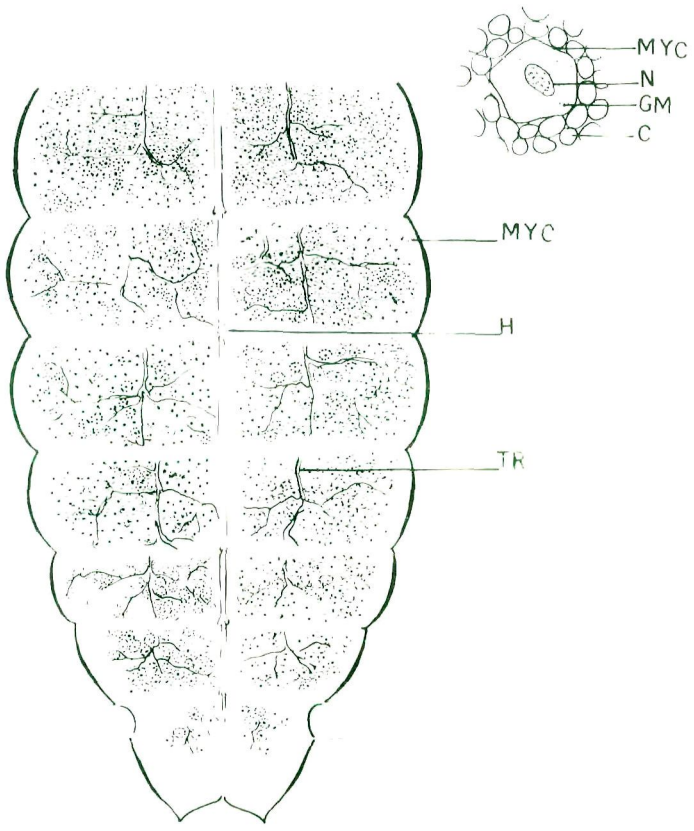


FIG. 17

Fig.18. Photomicrograph of a portion of the smear of fat body of *P. americana* showing microorganisms and nuclei stained with Giemsa stain.

C - Cytoplasm; GM - Granular microorganisms;
N - Nucleus; RM - Rod like microorganisms; and
V - Vacuole.

Fig.19. Photomicrograph of a longitudinal section of fat body of *P. americana* showing presence of mycetocytes.

C - Cytoplasm; EL - Enveloping layer; MYC - Mycetome and
N - Nucleus.

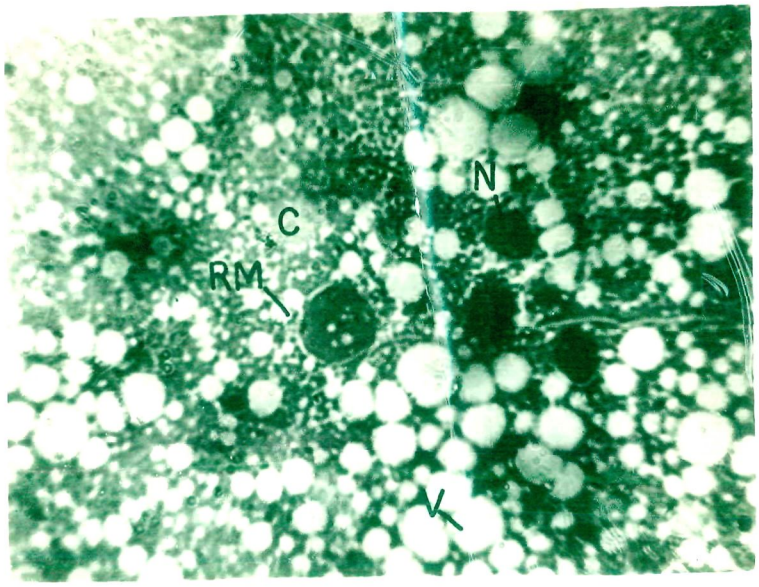


FIG. 18

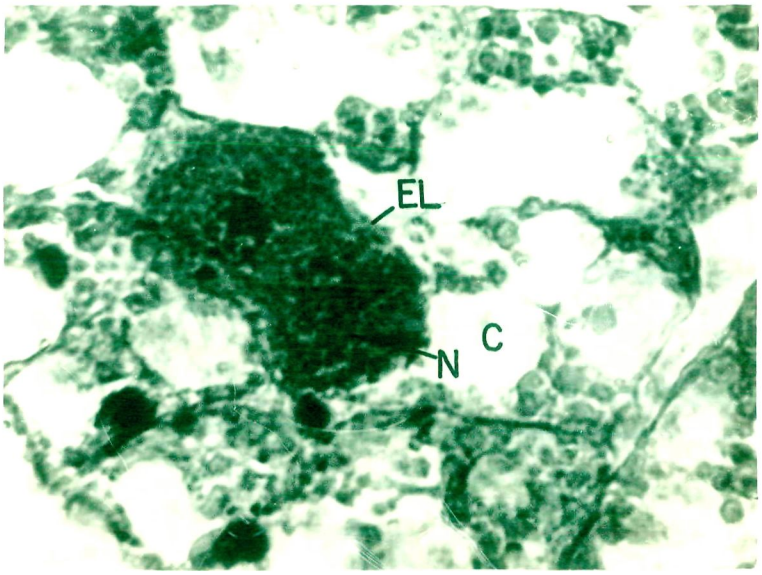


FIG. 19

Fig.20. Abdomen of adult of Sitophilus granarius L., dissected to show the anatomy of internal viscera and their relationship with intestinal caeca containing mycetocytes.

A - Anus; CA - Caeca; MI - Mid-intestine;
MT- Malpighian stubules and RM - Rectum.

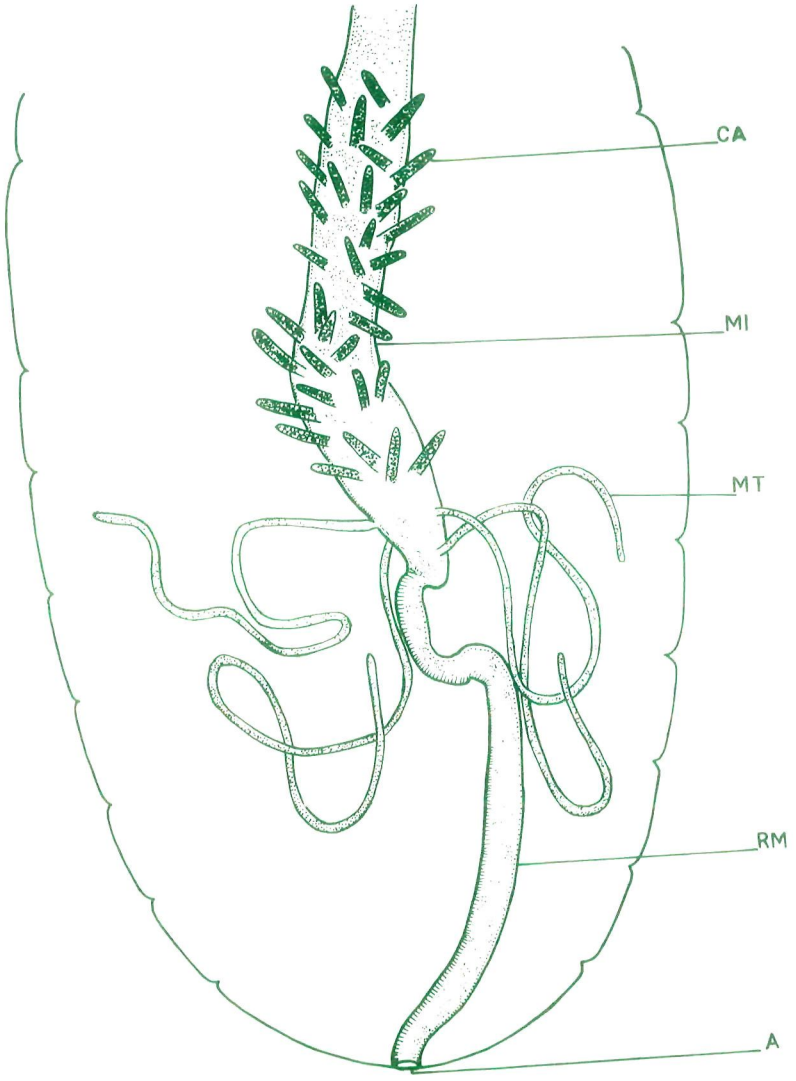


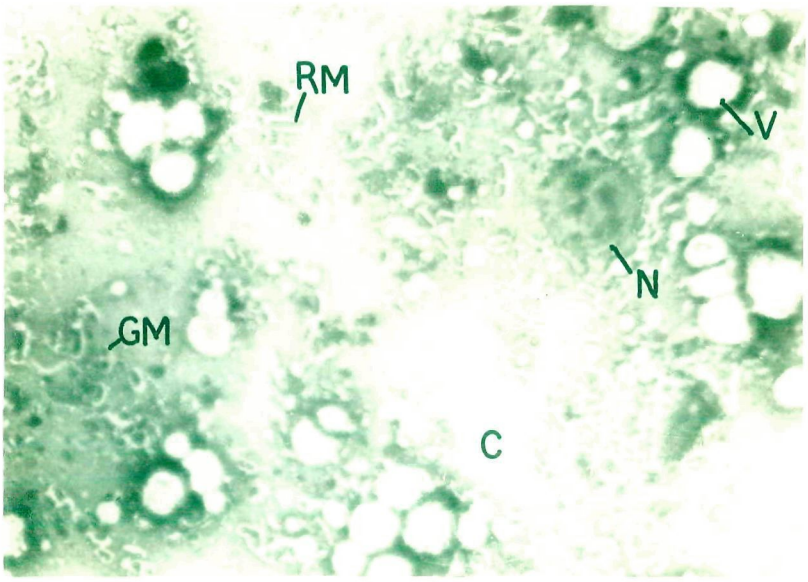
FIG. 20

Fig.21. Photomicrograph of a portion of the smear of ceaca of S. granarius showing microorganisms and nuclei stained with Giemsa stain.

C - Cytoplasm; GM - Granular microorganisms; N - Nucleus; RM - Rod like microorganisms and V - Vacuole.

Fig.22. Photomicrograph of a longitudinal section of ceeca of S. granarius showing presence of mycetocytes.

EL - Enveloping layer; MYC - Mycetocyte and N - Nucleus.



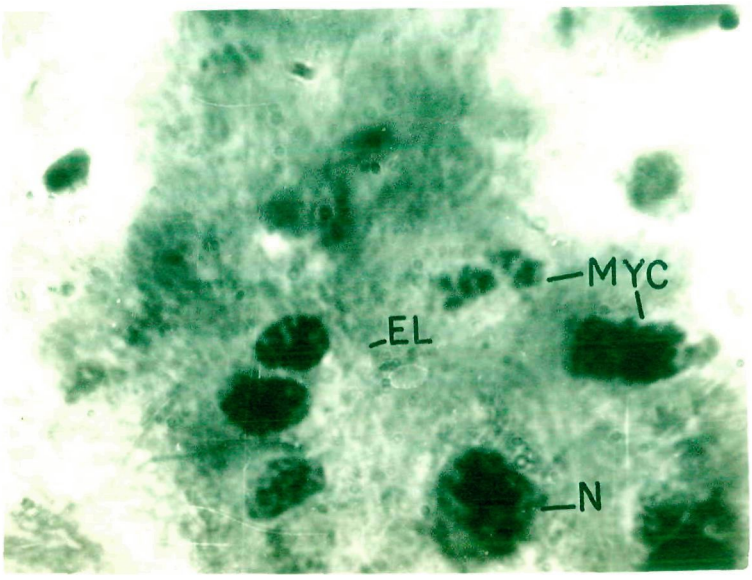


FIG. 22

Fig.23. Abdomen of adult male of *Cimex hemipterus* F. dissected to show the anatomy of internal viscera and their relationship with mycetome.

AG - Accessory gland; EJD - Ejaculatory duct; MYT- Mycetome; T - Testes and VD - Vas-deferens.

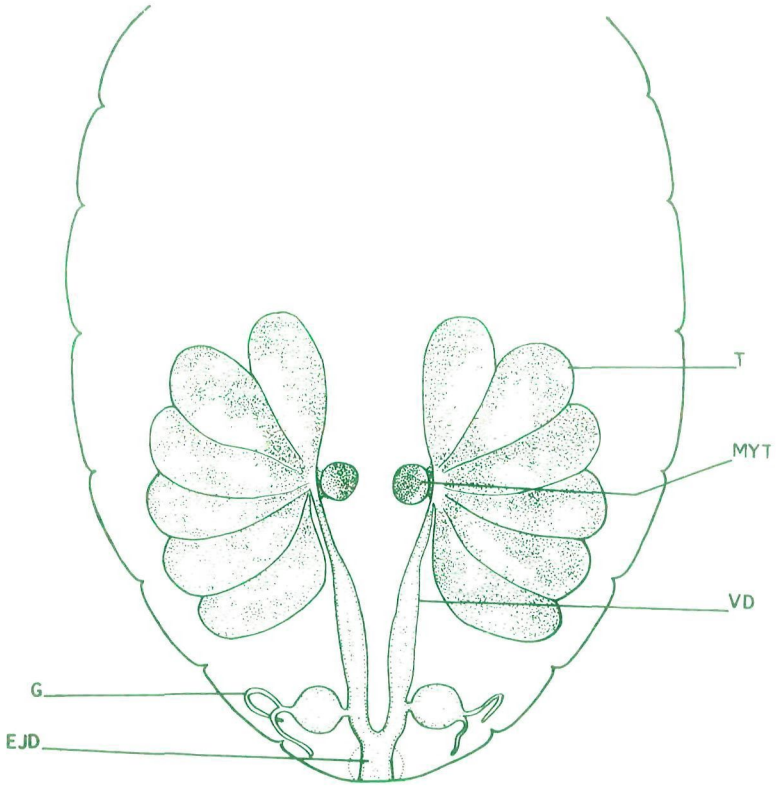


FIG. 23

Fig.24. Photomicrograph of a portion of the smear of mycetome of C. hemipterus showing microorganisms and nuclei stained with Giemsa stain.

C - Cytoplasm; GM - Granular microorganisms;
N - Nucleus; RM - Rod like microorganisms; and
V - Vacuole.

Fig.25. Photomicrograph of a longitudinal section of mycetome of C. hemipterus showing disintegration of the mycetocytes.

EL - Enveloping layer; MYC - Mycetocyte and
N - Nucleus.

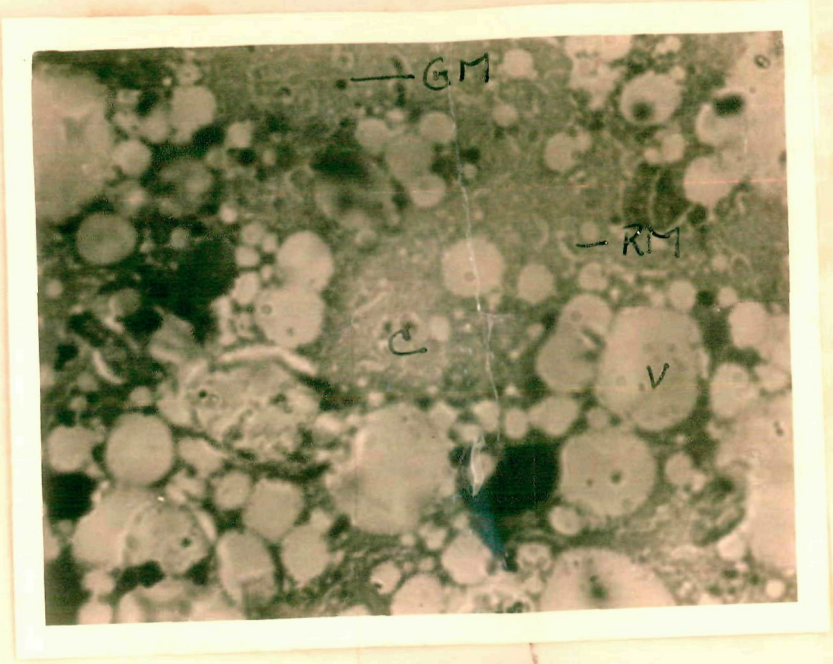


FIG. 24

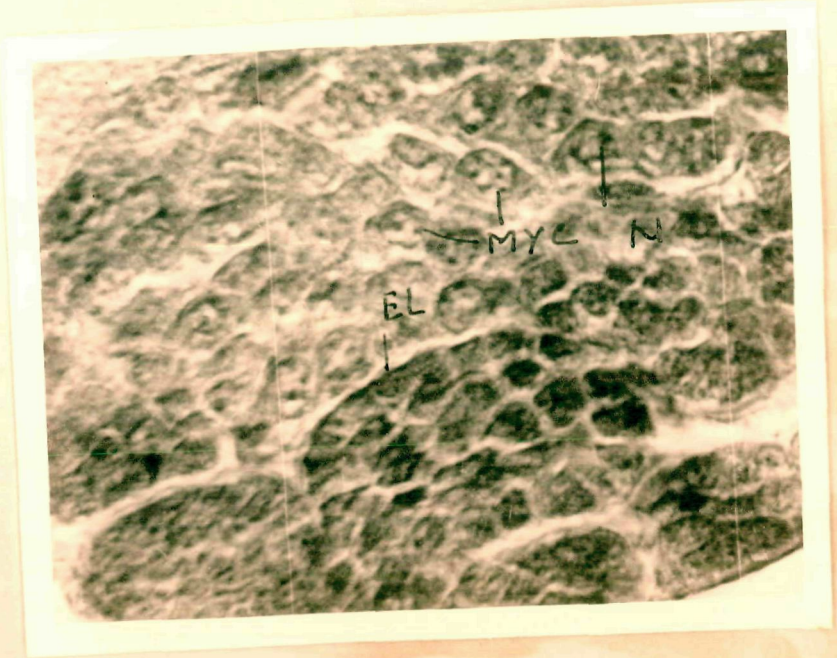


FIG. 25

Fig.26. Abdomen of Pediculus humanus capitis De Geer nymph dissected to show the anatomy of internal viscera and their relationship with the mycetome.

A - Anus; MI - Mid intestine; MYT - Mycetome; RM- Rectum and RP - Rectal papillae.

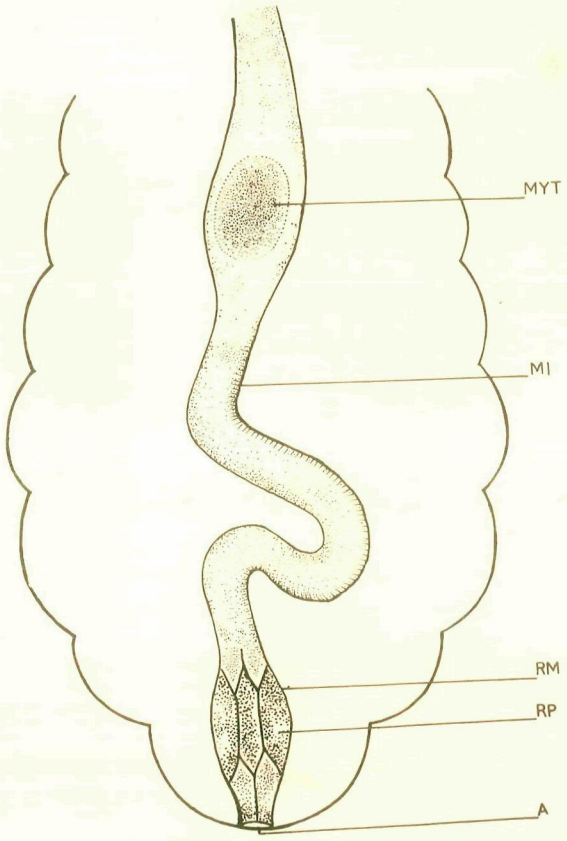


FIG. 26

Fig.27. Photomicrograph of a portion of the smear of mycetome of P.h. Capitis showing microorganisms and nuclei stained with Giemsa stain.

C - Cytoplasm; GM- Granular microorganisms;
N - Nucleus; RM- Rod like microorganisms and
V - Vacuole.

Fig.28. Photomicrograph of a longitudinal section of mycetome of P.h. capitis showing disintegration of the mycetocytes.

EL - Enveloping layer; MYC - Mycetocytes;
MYT- Mycetome and N- Nucleus.

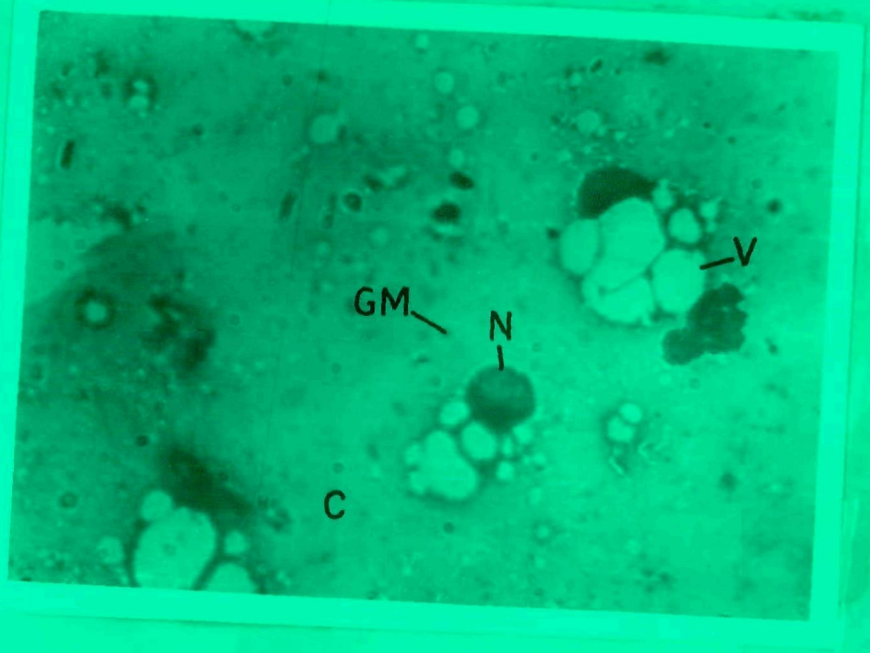


FIG. 27

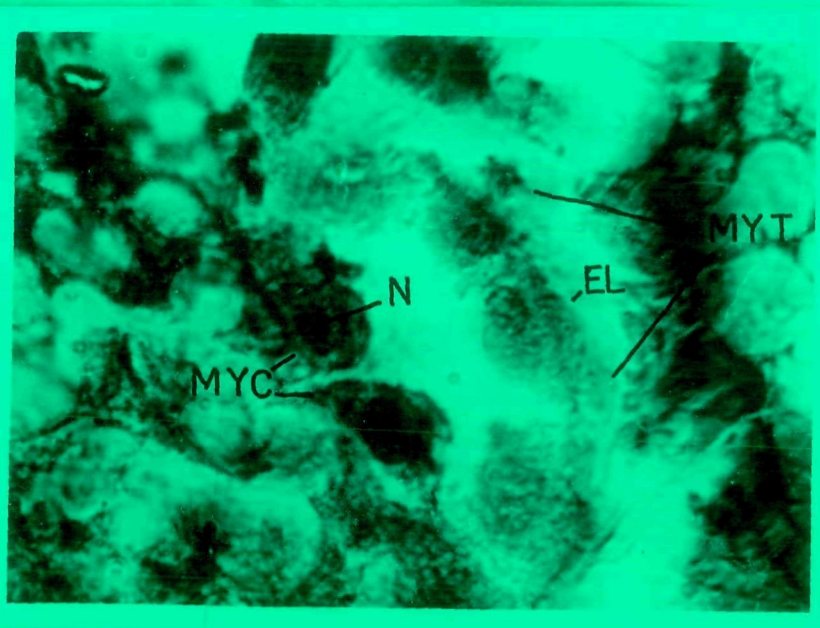


FIG. 28

V. HISTOCHEMISTRY OF THE MYCETOMES.

The presence of Carbohydrate, Protein and Lipids and Lipoproteins in the mycetocytes of P. perpusilla, I.clypealis and P. sexvittatus is given in the table (I-III). The distribution of these substances in the mycetocytes of these species is given below:-

CARBOHYDRATE:

a. Glycogen

The mycetocytes of P. perpusilla react strongly positive to periodic acid Schiff reaction (PAS). The glycogen takes up the stain in the form of granules in the cytoplasm. These granules are distributed homogenously in the mycetocytes (Fig.29). The nuclei and its membrane stain dark red, whereas granular shaped microorganisms around the nuclei stain purple. This indicates the presence of glycogen in the nuclei and microorganisms of the mycetocytes as well. On the other hand, I. clypealis shows weakly positive reaction to PAS. The cytoplasm takes up the stain irregularly in the form of patches of densely lightly stained areas. The nuclei appear like black spots, whereas, as the rod shaped microorganisms are stained purple red indicating the presence of glycogen in them (Fig. 30). In contrast to the mycetocytes of I. clypealis, the mycetocytes of P. sexvittatus show moderately positive to PAS. The staining reaction of the cytoplasm is homogenous.

The nuclear membrane is stained red whereas the nuclei and their chromatin material becomes black coloured showing the absence of glycogen. The granular shaped microorganisms around the nuclei stain purple red indicating the presence of glycogen (Fig. 31).

b. Acid mucopolysaccharides.

The mucopolysaccharides are absent from the mycetocytes of all the three species insects studied in the present investigation because neither cytoplasm and nor nuclei positively react to Alcian blue stain.

PROTEINS:

a. Glycoproteins.

The occurrence of this protein is confirmed by the Congo-red stain, the mycetocytes of P. pernyasilla react strongly positive to this stain. The cytoplasm stained brick red in the form of granules which are uniformly distributed. The nuclei and their membrane takes up dark red colour. Whereas microorganisms around the nuclei stain brick red indicating the presence of glycoproteins (Fig. 32). The mycetocytes of I. clypealis react less strongly than those of P. pernyasilla to Congo-red. The cytoplasm of these cells takes up brick red colour. However, the stain is not uniform and there are light stained patches as well. The nuclei and its membrane stain dark brown; the nuclear protein is marked with dark brown granules indicating the presence of glycoprotein. The rod shaped

microorganisms around nuclei take up brick red colour indicating comparatively less glycoprotein than in the nuclear content (Fig. 33). The occurrence of glycoprotein in the mycetocytes of P. sexvittatus is less marked than those of other sp. The cytoplasm is stained in patches of light brown areas. The nuclear contents is also stained in patches. Whereas the nuclear membrane is not stained showing the absence of glycoprotein in it. The granular shaped microorganisms around the nuclei take up light positive stain (Fig. 34).

b. Protein with tyrosine.

The demonstration of this substance is confirmed by the millon reaction, which gives brown colour. The mycetocytes of P. perpusilla show weakly positive to this reaction. The cytoplasm is stained homogenously in the form of granules. The nuclei do not show positive stain whereas granular shaped microorganisms show reddish brown indicating the traces of these proteins (Fig. 35). In I. clypealis the cytoplasm of the mycetocytes is moderately positive to millon reaction and stain is homogenously distributed thus indicating the presence of these proteins in the granular form. The nuclei became brown in colour and the microorganisms around the nuclei are pale coloured (Fig. 36) However, the mycetocytes of P. sexvittatus indicate positive reaction which is moderate

in intensity. The cytoplasm is homogenously stained in granular form. The microorganisms around the nuclei are yellow, whereas, the nuclei are stained dark brown indicating the presence of proteins (Fig. 37).

c. General protein.

The general protein indicates all sorts of protein which are stained with Hg. bromophenol blue stain. The mycetocytes of P. perpusilla are moderately positive, the distribution of general protein appears in the form of granule which are uniformly distributed stain. The nuclei are negative to this stain whereas the microorganisms around the nuclei are positively stained and indicated the localisation of proteins (Fig. 38). However, the mycetocytes I. clypealis reacted weakly positive to this stain and distribution of general protein is irregular. The nuclei and their membrane donot show positive staining (Fig. 39). However, the microorganisms around the nuclei are strongly positive to this stain. The mycetocytes of P. sexvittatus are moderately positive to this stain. The distribution of the protein is seen in the form of homogenously granules in the cytoplasm. The nuclei and their contents are negative to this reaction. However, the microorganisms indicated positive localization of general proteins (Fig. 40).

d. Ribonucleic acid (RNA)

The occurence of RNA in the mycetocytes of above mentioned insects is not confirmed with methyl green pyronin 'Y' stains show negative.

LIPIDS AND LIPOPROTEINS:

In the mycetocytes of P. perpusilla the distribution of these substance is irregular. The contration of these substance is fairly uniform in the nuclei however, there are more concentrations in the microorganisms (Fig. 41). The mycetocytes of I. clypealis has less concentration of lipids and lipoprotein than in those of P. perpusillar (Fig. 42). The cytoplasm has regular distribution. The contrast to the cytoplasm the nuclei show poor concentration of these substances. The rod shaped microorganisms and the nuclei have less marked concentration of these substances. But the mycetocytes of P. nexvittatus reacted strongly positive to Sudan black 'B' indicating higher concentration of lipid and lipoprotein which are distributed irregularly in patches in the cytoplasm. However, the nuclei have less concentration but the microorganisms are richer in lipids and lipoproteins than the nuclei (Fig. 43).

TABLE-I

HISTOCHEMISTRY ON THE MYCETOMES OF PYRILLA PERPUSILLA (WALKER.

S.No.	Staining technique	Reference	Fixative or Treatment	Indicated	Result
1.	Periodic acid schiff reaction.	McManus	Bouins fluid and N.B.F.	Carbohydrate containing proteins stains in various shades of purplish red, glycogen stains deeply.	+++
2.	Alcian blue	Steedman	Bouins fluid.	Acid mucopolysaccharides.	Q
3.	Congo red	Bennhold	Bouins fluid.	Clycoproteins (amyloid), material, brick red.	+++
4.	Millon reaction	Baker modification	Bouins fluid and N.B.F.	Proteins containing (Tyrosine) stained red, pink or yellowish red.	+
5.	Hg-Bromophenol Blue	Maize	Bouins fluid and N.B.F.	General proteins stained clear blue colour.	+
6.	Methyl green and Pyronin "Y"	Kurnick	Carnoys.	R.N.A., cytoplasm, bright red.	Q
7.	Sudan Black "B"	McManus	Bouins fluid and N.B.F.	Lipids and lipoproteins stained black or brownish black.	++

+++ = Strong reaction; ++ = Moderate reaction; + = Week reaction; and Q = No reaction; NB.F. = Neutral buffer formalin.

TABLE-II

HISTOCHEMISTRY ON THE MYCETOMES OF IDIOCERUS CLYPEALIS LETH

S.No.	Staining technique	Reference	Fixative or Treatment	Indicated	Result
1.	Periodic acid schiff reaction.	Mc'lanus	Bouins fluid and N.B.F.	Carbohydrate containing proteins stains in various shades of purplish red, glycogen stains deeply.	+
2.	Alcian blue	Steedman	Bouins fluid.	Acid mucopolysaccharides.	O
3.	Congo red	Bennhold	Bouins fluid.	Glycoproteins (amyloid), material, brick red.	++
4.	Millon reaction	Baker modification	Bouins fluid and N.B.F.	Proteins containing (Tyrosine) stained red, pink or yellowish red.	++
5.	Hg-Bromophenol Blue	Malza	Bouins fluid and N.B.F.	General proteins stained clear blue colour.	+
6.	Methyl green and Pyronin "Y"	Kurnick	Carnoys.	R.N.A., cytoplasm, bright red.	O
7.	Sudan Black "B"	McManus	Bouins fluid and N.B.F.	Lipids and lipoproteins stained black or brownish black.	+

+++ = Strong reaction; ++ = Moderate reactions + = week reaction; and O = No reaction; N.B.F. = Neutral buffer formalin.

TABLE-III

HISTOCHEMISTRY ON THE MYCETOMES OF PTYELUS SEXVITTATUS WALKER.

S.No.	Staining technique	Reference	Fixative or Treatment	Indicated	Result
1.	Periodic acid schiff reaction.	McManus	Bouins fluid and N.B.F.	Carbohydrate containing proteins stains in various shades of purplish red, glycogen stains deeply.	++
2.	Alcian blue	Steedman	Bouins fluid.	Acid mucopolysaccharides.	O
3.	Congo red	Bennhold	Bouins fluid.	Glycoproteins (amyloid), material, brick red.	+
4.	Millon reaction	Baker modification	Bouins fluid and N.B.F.	Proteins containing (Tyrosine) stained red, pink or yellowish red.	++
5.	Hg-Bromophenol Blue	Maiza	Bouins fluid and N.B.F.	General proteins stained clear blue colour.	++
6.	Methyl green and Pyronin "Y"	Kurnick	Carnoys.	R.N.A., cytoplasm, bright red.	O
7.	Sudan Black "B"	McManus	Bouins fluid and N.B.F.	Lipids and lipoproteins stained black or brownish black.	+++

+++ = Strong reaction; ++ = Moderate reaction; + = week reaction; and O = No reaction; N.B.F. = Neutral buffer formalin.

Fig.29. Photomicrograph of longitudinal section of the mycetome of Pyrilla perpusilla stained with PAS showing the distribution of glycogen in the mycetocytes.

Fig.30. Photomicrograph of longitudinal section of the Idiocerus clypealis stained with PAS showing the distribution of glycogen in the mycetocytes.

Fig.31. Photomicrograph of longitudinal section of mycetome of Ptyelus sexvittatus stained with PAS showing the distribution of glycogen in the mycetocytes.

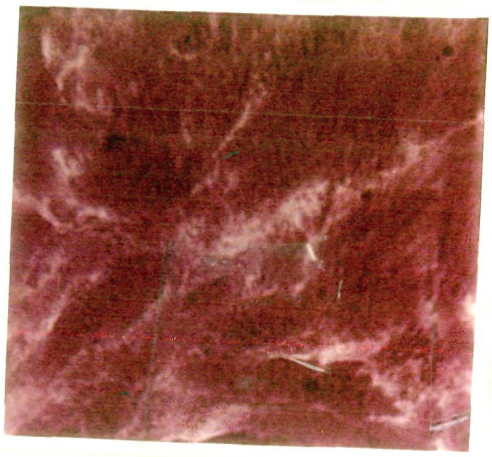


FIG. 29

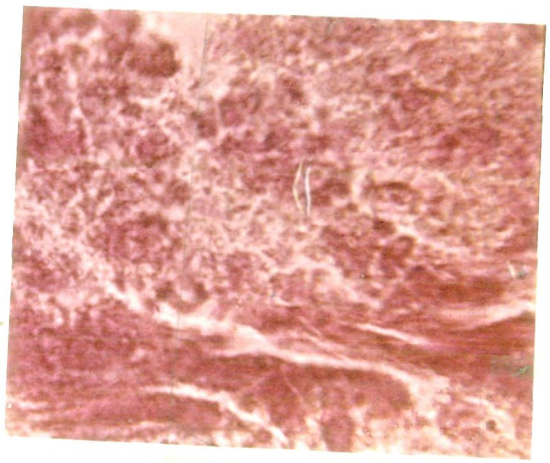


FIG. 30

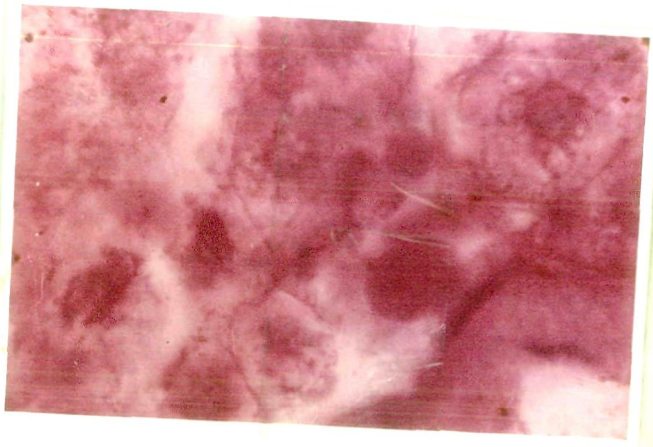


FIG. 31

Fig.32. Photomicrograph of longitudinal section of the mycetome of P. perpusilla stained with Congo-red showing the distribution of glycoprotein (Amyloid) in the mycetocytes.

Fig.33. Photomicrograph of longitudinal section of mycetome I. clypealis stained with Congo-red showing the distribution of glycoprotein (amyloid) in the mycetocytes.

Fig.34. Photomicrograph of longitudinal section of mycetome of P. sexvittatus stained with Congo-red showing the distribution of glycoprotein (Amyloid) in the mycetocytes.

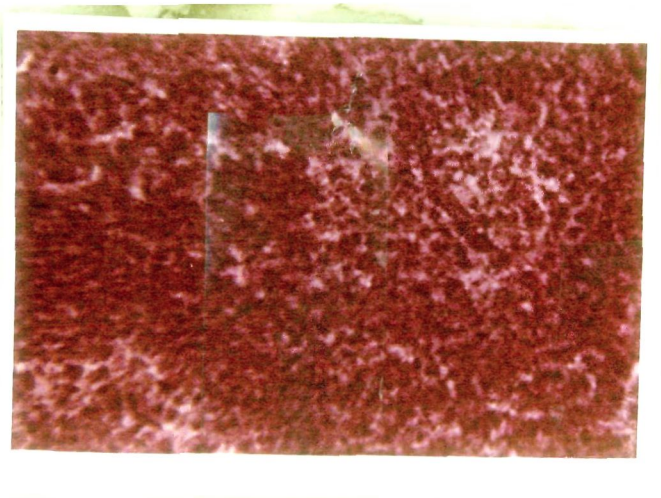


FIG. 32

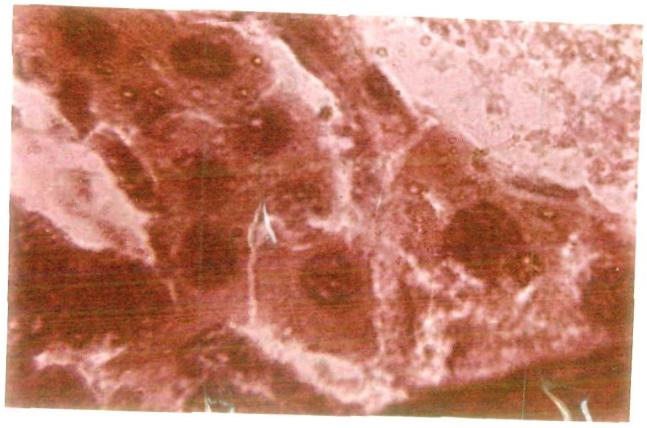


FIG. 33

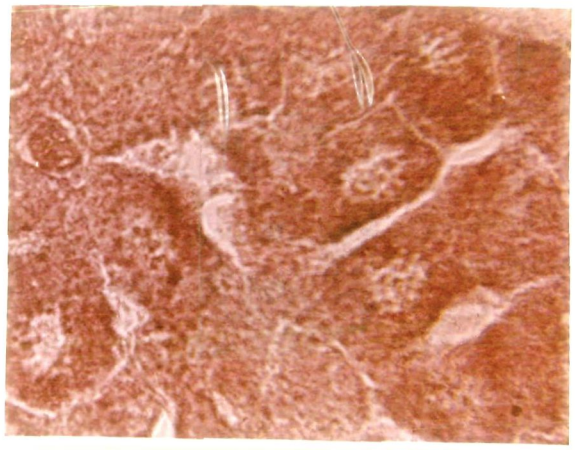


FIG. 34

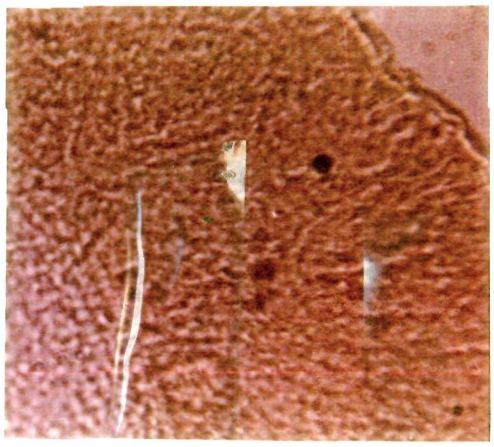


FIG.35

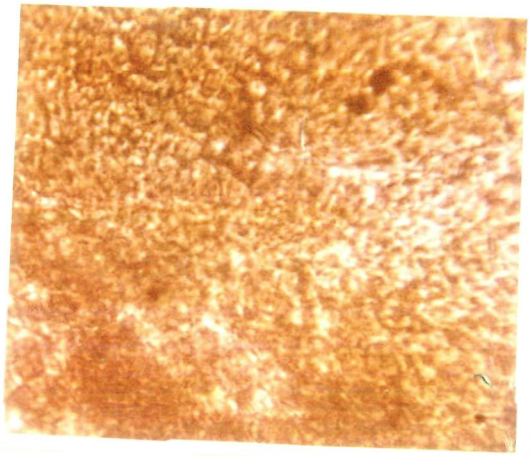


FIG.36

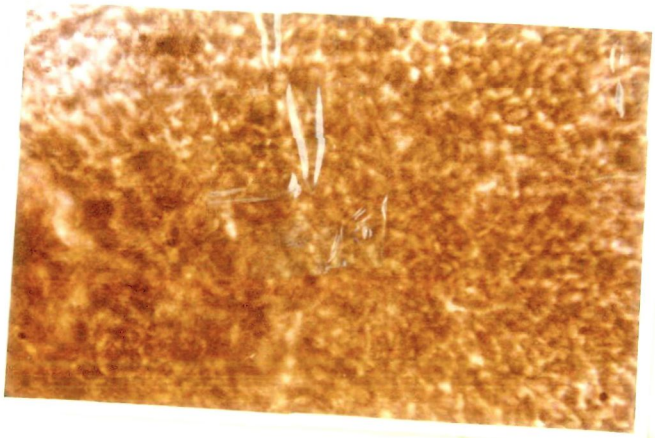


FIG.37

Fig.35. Photomicrograph of longitudinal section of the mycetome of P. perpusilla stained with Millon reaction showing the distribution of protein with tyrosin in the mycetocytes.

Fig.36. Photomicrograph of longitudinal section of the mycetome of I. clypealis stained with Millon reaction showing the distribution of protein with tyrosine in the mycetocytes.

Fig.37. Photomicrograph of longitudinal section of the mycetome of P. sexvittatus stained with Millon reaction showing the distribution of protein with tyrosine in the mycetocytes.

Fig.38. Photomicrograph of longitudinal section of the mycetome of P. perpusilla stained with Hg Bromophenol blue showing the distribution of general protein in the mycetocytes.

Fig.39. Photomicrograph of longitudinal section of the mycetome of I. clypealis stained with Hg Bromophenol blue showing the distribution of general protein in the mycetocytes.

Fig.40. Photomicrograph of longitudinal section of the P. sexvittatus stained with Hg Bromophenol blue showing the distribution of general protein in the mycetocytes.

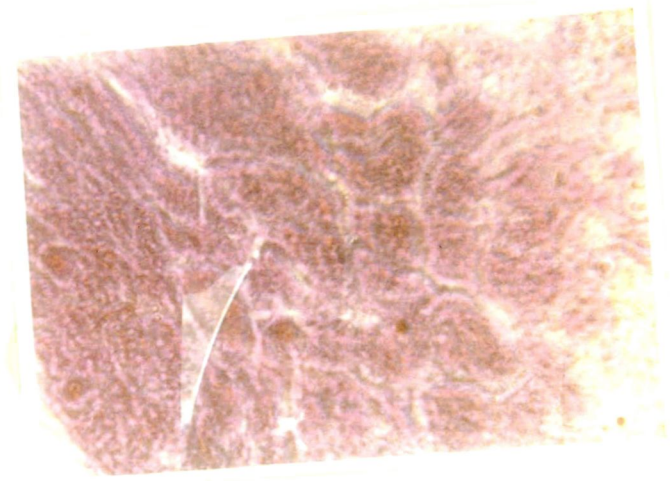


FIG. 38

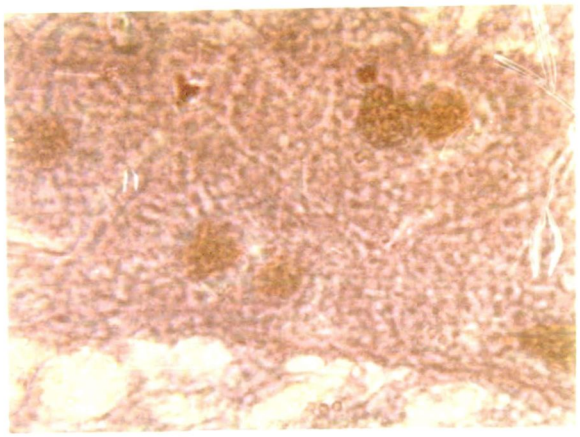


FIG. 39

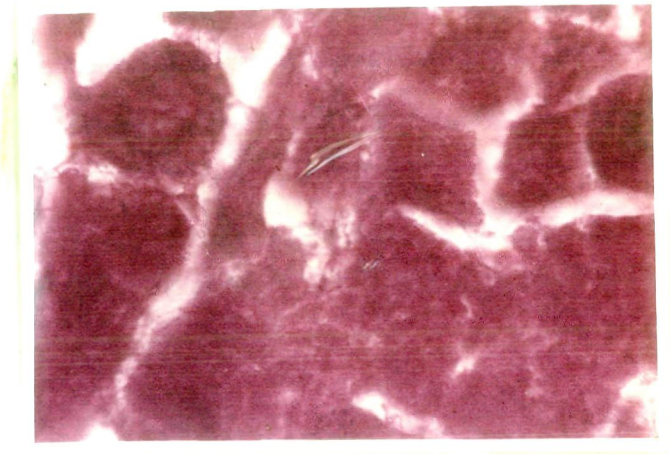


FIG. 40

Fig.41. Photomicrograph of longitudinal section of the mycetome of P. perpusilla stained with Sudan Black B showing the distribution of Lipids and Lipoprotein in the mycetocytes.

Fig.42. Photomicrograph of longitudinal section of the mycetome of I. clypealis stained with Sudan Black B showing the distribution of Lipids and Lipoprotein in the mycetocytes.

Fig.43. Photomicrograph of longitudinal section of mycetome of P. sexvittatus stained with Sudan Black B showing the distribution of Lipids and Lipoprotein in the mycetocytes.

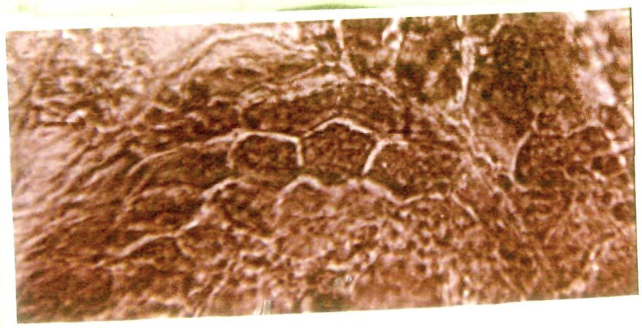


FIG. 41

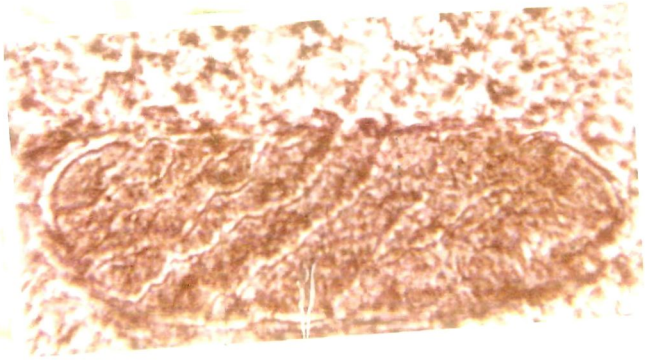


FIG. 42

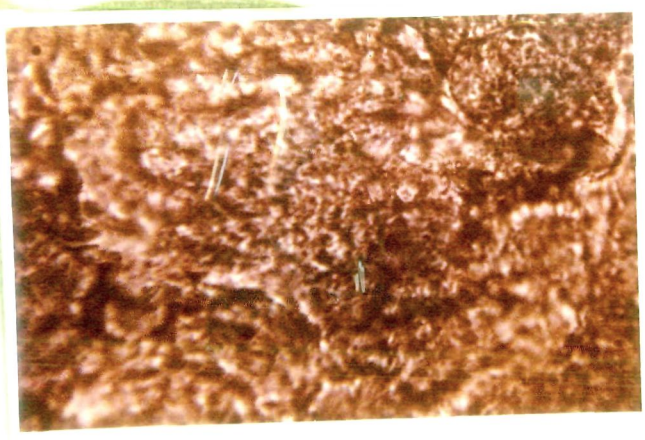


FIG. 43

VI- DISCUSSION AND CONCLUSION

A careful study of the dissected insects shows that the mycetomes and mycetocytes very much vary in shape. They are tubular and 'Y' shaped, long and thread like, trilobed and oval or pear shaped in Pyrilla perpusilla Walker, Idiocerus clypealis Leth, Ptyelus sexvittatus (Walker), Cimex hemipterus ', respectively and oval in the nymph of Pediculus humanus capitis Deseer. The mycetocytes of Periplaneta americana L, are embodded in fat bodies and that o' Gryllophilus grandrius (L) in the 'U' shaped intestinal caeca. The colour of the mycetomes and mycetocytes is highly variable. It is brown in P. perpusilla, pale-yellow in I. clypealis, red in P. sexvittatus, white in C. hemipterus and G. grandrius, cream in P. americana and yellow in P.H. capitis.

The mycetomes are paired in P. perpusilla, I. clypealis, P. sexvittatus and C. hemipterus. The discovery of paired nature of mycetomes in the insects is in agreement with the finding of Buchner (1933 and 1939) on Cimex lectularius, Oxytoma sp., Erytranion sp., and Pretanion sp.,

Similar condition has been reported by Mansour (1934) in Rhizopertha dominica, Bostrycho plites, Koch (1936) in Lyctus linearis and Schneider (1940) in Mysius sp. On the

other hand, in P.h. capitis the mycetome is unpaired. This gets support from the findings of Aschner (1934) in P.h. Capitis. In the case of P. americana the mycetocytes are scattered in the haemocoel in close association with the ovaries and testes. In S. granarius these are lodged inside the intestinal caeca and gets support from the findings of Goir (1936) in P. americana, Murry and Peig (1934) followed by Musgrave et al. (1932 and 1964) on S. granarius respectively.

The mycetome of P. perpusilla is found lying on the dorsum of proctodaeum. In I. clypealis, P. sexvittatus and C. hemipterus it is present on either side of the alimentary canal in abdominal segment. In P.h. Capitis the mycetome is located on small organ known as stomach disk.

It appears from the observation that the mycetomes in P. perpusilla, I. clypealis, P. sexvittatus, C. hemipterus and P.h. capitis have no connection with the internal viscera of the host. Whereas in P. americana and S. granarius the mycetocytes penetrate inside the fat bodies and intestinal ceaca respectively. It has been observed that the mycetome after the separation from the body of the host, when kept in distilled water show sign of disintegration after one to two hours. But in the case of normal saline they will remain alive in P. perpusilla, I. clypealis, P. sexvittatus, C. hemipterus, P. americana, S. granarius and P.h. Capitis reveals the

presence of different shaped microorganisms staining deep red, reddish blue or pink with Geimsa stain. The nuclei of the mycetocytes also take up similar colour with Geimsa stain. The microorganisms are scattered all over the smear. In the case of P. perpusilla, P. sexvittatus, P.h. capitis and P. americana the bulk of the microorganisms are of granular shape. Besides, a few rod shaped microorganisms are also present. While in I. clypealis, C. hemipterus and S. granarius the rod shaped microorganisms are in abundence scantly interposed with few granular shaped microorganisms.

Similar polymorphism in microorganisms has been recorded by Brooks (1963) and Khan (1972) in cockroach and P. perpusilla respectively. They suspect influence of the variety of microorganisms on the host physiology specially in respect to age and sex. Koch (1956) and Cleveland (1959) suggest that the hormones are involved in the cyclic exchange of the microorganisms.

The present writer suspects increase in number of microorganisms with the age of the host but it appears that the number of microorganisms in the mycetomes of adult host does not grow beyond a certain limit. Glaser (1920) also made a similar observation in the insects. According to him some secretions appear to inhibit the growth of the microorganisms beyond a reasonable limit; otherwise the host will be killed. In the present investigation the nuclei assume

giant size which is likely to be a stage of pycnosis. The suspected disintegration may be attributed to the microorganisms. Mehdihassan (1957) considers similar condition as a pathological symptom. Such large nuclei have also been reported by Brooks and Richards (1955) in cockroaches and by Baudisch (1956 and 1959) in some other insects.

The cytoplasm of the mycetomes in the smear of insects under study takes a reddish tinge. Mehdihassan (1957) also reported a similar conclusion in coccids and pointed out that this is due to the activity of microorganisms present there. In P. pernusilla, L. clypealis, P. saxvittatus, C. hemipterus, P. americana, S. granarius and P.h. Capitis a number of vacuoles are found containing microorganisms. There was a slight suspicion in the mind of the present writer impression of considering the rod shaped micoorganisms as mitochondria. This fact that these microorganisms persist inspite of the treatment with Bouins fluids, confirms that the microorganisms are certainly not mitochondria. This fact that the intracellular inclusions are definitely the microorganisms is also supported by Steinhaus (1946). Similar conclusions have been made by Buchner (1953), Wigglesworth (1953), Mehdihassan (1957) Grosson and Threadgold (1960), Buch and Chapman (1961) and Musgrave et al (1962) in different insects. The metabolic activity takes place within the mycetome due to the presence of microorganisms, hence oxygen is supplied to the mycetomes

through a complicated tracheal ramification of the host. Vichanco (1924) while working on aphids also observed similar profused tracheal supply to the mycetomes. Same is the observation of Lysenko and Salama (1969).

A study of the longitudinal section of mycetome of P. perpusilla, T. clypealis, P. sexvittatus, C. hemipterus and P.h. capitis shows that they are externally covered by an enveloping layer. The mycetome is found to contain scattered mycetocytes adjacent to the covering layer. The mycetocytes are more or less arranged in longitudinal columns, while in the case of P. americana each mycetocyte is easily distinguished from the fatty tissue by an enveloping layer. In S. granarius the intestinal caeca contains mycetocytes enveloped by covering membrance and in association with its epithilum.

Each mycetocytes has a centrally placed nucleus. The presence of giant nuclei confirms the occurrence of pycnosis in the mycetocytes. Such enlarged nuclei have also been reported by Brooks and Richards (1955) in cockroach. The nuclei of mycetocytes are observed to undergo disintegration followed by transformation of mycetocytes into syncytial form. This type of disintegration of mycetocyte has been reported by Mansour (1934), Naidu (1945) and Mehdihassan (1957).

The cytoplasm of the covering layer of the mycetocytes shows that it is extremely thin with distantly placed island of nuclei. Furthermore it is like peritonial layer.

The present histochemical observations on the mycetome of P. perpusilla, I. clypealis and P. sexvittatus indicate that there is negative reaction to methyl green pryonin 'Y' stain which confirms the absence of RNA both in the cytoplasm and nuclei of these mycetocytes. Therefore it is very likely that proteins observed in these mycetocytes are not synthesized locally but they are stored in these microorganisms. The source of the transport of these proteins may be haemolymph which surrounds the mycetomes in all the three species. However, in the P. perpusilla the organisation of the mycetome is closely related to the wall of proctodium (Fig. 1,2). Whereas in I. clypealis and P. sexvittatus the mycetomes are suspended into the haemocoel and donot rest in the alimentary canal (Fig. 7,8,12 and 13). The sites of the mycetome do not suggest any physiological relationship with the digestive organs. Proteins have also been found in the mycetocytes of Pear psylla, Psylla pyricola Foerst (Chang and Musgrave 1969). RNA has also been reported by Chang and Musgrave (1969). The glycogen has been found both in the nuclei and as well as in the cytoplasm of the mycetocytes of all the species in the present investigation. Although the study was based on the qualitative methods variation in the concentration of glycogen in different mycetocytes may be appreciated. However, the cytoplasm appears to be the main site of concentration of glycogen in all the species. Further this substance has been

most distinctly recognised than the other substances. As regards the synthesis of glycogen in the mycetocytes it is only premature to confirm. However, like other tissues of the haemocoel such as haemocytes and pericardial cells etc. mycetocytes may also serve as storage cells for glycogen as well.

Lipids and lipoproteins are generally stored in fat body cells of the insect. It is a well known fact the the metabolism of lipids and lipoproteins generally takes place in the fat body cells of the insect. However, in some insect the haemocytes also play the role of storage cells for the lipids. Such haemocytes have been identified as either adipocytes or adipohaemocytes in different insects. Notwithstanding the fact that the occurrence of adipohaemocyte in the present investigation it is further likely that the lipids and lipoproteins of the mycetocytes are stored products of metabolism.

The present data on the histochemical presence of glycogen, glycoproteins, protein bond with tyrosine, general protein and lipids and lipoproteins in the mycetocytes of P. perpusilla, I. clypealis and P. sexvittatus are additions to the knowledge on the mycetome in general. The significance of these substances to host can only be determined by further experimental investigation.

P A R T - B

I- INTRODUCTION

The difficulties in culturing the microorganisms associated with mycetomes were surmounted through the workers of Mercier (1906 and 1907), Glaser (1920 and 1930), Hertig (1921), Gropengiesser (1925), Metiz (1926), Gier (1931), Eklom (1931), Hoover (1945), Steinhaus (1946 and 1946), Pant and Fraentel (1954) and Daines (1956). All of them claimed that all the microorganism isolated from mycetomes could be easily grown on artificial media. Brooks (1963), on the other hand, pointed out that the intracellular symbiotes (the word symbiotes and symbionts used interchangeably in the literature) were not amenable to the various culture techniques and that the apparent growth of the symbionts in a few cases were due to the contamination of culture media. Similar claim ealier made by Steinhaus (1949), that symbiotes could exist as free-living organism. He further claimed that thir possible mode of existence perhaps represented an independent relationship between microorganism and the host. Puchta (1955) after making repeated efforts was successful in isol ting symbiotes from the mycetomes of Pediculus vestimenti and cultured them on Blood agar. Potter (1955) and Newig and Schwart (1966) also succeeded in culturing the microorganisms from the mycetomes of Haematopinus suis and they remained alive without any sign of reproduction for a few days in isotonic cultures.

Blood sucking insects such as bedbugs, lices and testes fly were found to harbour symbiotic microorganisms Wigglesworth, (1952) and Brooks (1963) as early as 1926 suggested that mycetomes of certain aphids harboured two different types of microorganisms. Similar views were expressed by Carter (1935 and 1933), and Buchner (1955) and Le Dalance and Musgrave (1963) for coccids and certain other insects.

Similarly, the views pertaining to nature of symbionts was not clear. Carter (1936) stated that mycetomal symbionts of mealy bugs were injurious to the host. Pant and Frankel (1950), Lanham (1952) Trager (1952), Buchner (1953), Wigglesworth (1953) Goodchild (1955), Baines (1956) provided conclusive evidence that there existed a mutually beneficial relationship (mutually symbiotic) between the micro-organism on one hand and the insect host on the other. According to Musgrave and Miller (158) granary weevil, S. granaria harboured large number of microorganisms some of which were also thought to be beneficial to the host, insect. Cabrani (1970) had successfully cultured the bacteria from the mycetomes of Sitophilus in nutrient broth agar media which was found to be similar to Bacillus circulans.

Electron microscopic studied by Buchner (1953), Wigglesworth (1953), Gresson and Threadgold (1960) Buch and Chapman (1961) and Musgrave et al. (1963) provided further proof to this view.

Moreover, Koch (1959 and 1960) detected DNA in the thread like bacteria from Camponotus ligniperda but not from bacteria isolated from Sitophilus. The existence of DNA was, however, provided by Musgrave et al (1962) and Grinyner and Musgrave (1966).

Steinhaus (1947) was not very sure about their role in the intestinal tract of certain insects. Although Pierantoni as early as 1910 pointed out that symbiotic bacteria converted sugar absorbed by plant sucking insects such as aphids coccids, psylids into simpler substances which were absorbed by insects. Wigglesworth (1962) observed that digestive enzymes were not secreted in these segments of insect intestine where bacterial symbionts were present. Consequently digestion failed to take place in that region of intestine.

Wigglesworth (1936) provided evidence that symbiotic bacteria provided an endogenous source of vitamins and other accessory growth factor to insect hosts resulting that some of the insects could live in a restricted or highly specialised diet which otherwise might be even deficient in certain respects. Frankel and Bellwott (1943) concluded that intracellular microorganisms provided a source of vitamins to non-blood sucking insects such as Lasioderma serricorne and Stegobium paniceum. Garbner (1954) suggested that bacterial symbionts of the family Anobiidae metabolised vitamins which were required as growth factors. Gumpart and Schwartz (1963)

demonstrated that microbial symbionts belonging to family Triatomidae also synthesized vitamins.

Symbionts of Homopterus insects played an important role in nitrogen metabolism and were capable of breaking down urates (Sulc, 1910). Microbial degradation of urates occurred in aphids which lacked malpighian vessels and in coccid where they were degenerated (Buchner, 1912). Frank (1954 and 1956) provided experimental evidence about the presence of bacterial symbionts in organs of excretion including fat bodies of the Blattidae. Selmair (1962) and Pierre (1964) observed a definite increase of urate content of Blattidae when symbionts were removed. Moreover, the Blattidae symbionts could also be successfully cultured in media containing uric acid and thus supporting the above hypothesis. Pierre (1962) earlier demonstrated the presence of uricase, capable of oxidising uric acid into allantoin both in cultures of the symbionts of Leucophaea maderae and in the fat bodies of aposymbiontic specimens. He further claimed that symbiotic bacteria of cockroaches synthesized vitamin C which apparently was required by microorganisms but was of no significance to the host. Another role attributed to the symbionts of Pseudococcus citri in culture was their capability of assimilating atmospheric nitrogen. This organism was also able to utilize urea and allantoin which according to Wigglesworth (1950) appeared in

malpighian vessels as final products of purine metabolism and aminoacids (Fink, 1962 and Kohler and Schwartz, 1962). P. citri failed to utilize uric acid Xathine, quanine and adenine when the pH of the culture medium tended towards alkalinity. This also held true from the organisms cultivated from P. maritimus except that the organisms from P. maritimus failed to assimilate atmospheric nitrogen and urea and allantoin could only be utilized in the presence of carbohydrates. The symbionts of aphids such as (Doralis (Aphis) saliceti, Megoura viciae (Fhrhardt, 1962, 1963 Cavariella aegopodii) could also utilize urea, uric acid and hippuric acid provided ± the Bacterium aphidinum which was isolated by Schoel (1934), is identical with the symbionts in the mycetomes of the leaf lice. Toth (1952) reported that symbionts from Aphis brassicae failed to assimilate atmospheric nitrogen in culture medium containing urea and uric acid. When using labelled nitrogen Toth (1959) failed to confirm his earlier findings.

A new approach to mycetomal research was initiated by Koch (1936) who observed bacterial inhibition in the fat bodies of Periplaneta orientalis when trypaflavin was injected into the body cavity of the insects. Inhibitional effect on mycetomal bacteria with sulpha drugs such as sulfanilamide, sul athiozol, sul adiazine and Na-sulfanilate was reported by Brues and Dunn (1945). The discovery of antibiotics opened a new filed in symbionts research. Highly satisfactory

results were obtained with the use of antibiotics such as penicillin, chloromycetin, streptomycin, terramycin, hostacyclin with aposymbiotic animals. Chlorotetracyline proved to be highly effective against Blattella germanica (Brooks, 1954 and Brooks and Richards, 1955), P.orientalis Franks (1954 and 1956), Rhizopertha domanica and Oryzaephilus surinamensis Huger (1956), Calandra (Sitophilus) granaria and oryzae) Schneider (1953). However, the symbionts of Triatoma infestans in pure culture were little effected by Chlorotetracycline and chloromphenicol (Geigy et al., 1954). Malke (1954 a, b) in Schwartz laboratory was able to influence permanently the symbiotic bacteria of different Blattidae by using lycozyme. In hypotonic and isotonic media the bacteria disingegrated however, when the medium contained 20 per cent sucrose they were transformed from rods to spheres. This lead Malke to conclude that it was possible to obtain aposymbiotic animals as a result of action of lysozyme. Behrenz and Technau (1959) while using perfused wood with sulphanilamide (4-aminobenzolsulfonamide) were able to eliminate the symbionts of larvae of Anobium punctatum which thrived in wood. Recently Jurzitza (1963) got negative result for the symbionts from L. serricorne.

Antibiotic resistance has been observed in some of the symbiotic bacteria from insect host. Brooks and Richards (1955) reported that the females of B. germanica were able to prolong

their life span, while being fed on a diet containing 0.1 per cent aureomycin during the course of the antibiotic treatment. The study further indicated that the adults were never found to be symbiont free, though the offspring from such insects were all aposymbiontic. Antibiotic-resistant bacterial population was obtained for the mycetomes of P. orientalis after treating with streptomycin or with higher doses of chlorophenical (chloromycetin). But in most other cases, the antibiotic treatment of mycetomes completely eliminated the symbiotic bacteria from insect host.

The appearance of antibiotic resistant population after treatment with various drugs has been explained by Frank (1956) as a phenomenon of 'acquired' as opposed to 'internal resistance'. Hugger (1956) also supported the above findings by describing the resistant symbionts in B. dominica.

It is evident from the foregoing account that little is known about the microorganisms of the mycetomes associated with Pyrilla perpusilla Walker, Idiocerus clypealis Leth and Ptyelus sexvittatus Walker. The present studies were carried out with the view to isolate the microorganism associated with the above insects, to study certain aspects of microbial metabolism and to determine the effect of some of the antibiotics on the growth of mycetomal bacteria in culture.

II- MATERIALS AND TECHNIQUES

ISOLATION OF BACTERIA FROM THE MYCETOMES:

For isolating the mycetomal bacteria from P. perpusilla, I. clypealis and P. sexvittatus the adult insects were etherised. The legs and wings were removed and the remaining portion of the body sterlized 1: 1000 solution of mercuric chloride in 80 per cent ethanol. They were then rinsed in sterile distilled water and latter transferred to sterile normal saline. The insect body was dissected under sterile condition under a steroscopic microscope and the mycetomes after removal were transferred to sterile saline solutions contained in 1 ml tubes, it was then macerated by means of a sterile sharp needle. It was then transferred to cultured tube containing sterile nutrient broth. The cultured tube was then incubated at a 0°, 4°, 25°, 37°C and at room temperature for a period of 24 hours. The bacteria from all such tubes showing turbidity were subsequently plated on nutrient agar, potato agar and nutrient gelatin, and incubated at 37°C. Individual colonies from the above plates were transferred to nutrient agar slants and incubated at 37 C for 24 hrs. The slants were later stored in a refrigerator at 4 C.

PREPARATION OF MEDIA:

Nutrient broth was prepared by dissolving 5.0 g of peptone; 3.0 g of beef extract; 1.0 g of sodium chloride in 1000 ml of distilled water at pH 7.0. The medium was dispensed in 15.0 ml portion in screw cap culture tubes and sterilized by autoclaving at 121 C (15 lbs) for 15 mts.

Potato-gar medium was prepared by adding 1000 g of peeled and washed potato pieces to 300 ml water. The mixture was steamed in an autoclave for one hour. The extract was subsequently filtered through cotton and the volume was made upto 1000 ml.

Nutrient agar medium was prepared by dissolving 5.0 g peptone; 3.0 g beef extract; 1.0 g sodium chloride and 20.0 g agar in 1.0 litre of distilled water.

Nutrient gelatin medium was prepared by dissolving 3.0 g of beef extract; 5.0 g of peptone and 120.0 g of gelatin in 1.0 litre of distilled water. The mixture was heated in water bath at 65 C. The pH was adjusted at 7.0.

Methyl red (MR) and Acetyl-methyl carbinol (Voges Proskauer;VP) test media were made dissolving 7.0 g of peptone and 5.0 g of dibasic potassium phosphate in 1.0 litre of distilled water at a pH of 7.2.

Nitrate broth medium was prepared by dissolving 1.0 g of peptone; 0.05 g of potassium; 0.05 g of sodium chloride in 1.0 litre of distilled water.

Indol medium was prepared by dissolving 0.5 g of trypto phane (Bacto) and 5.0 g of sodium chloride in 1.0 litre of distilled water. The pH was adjusted at 7.0.

Hydrogen sulphide test medium was prepared by dissolving 15.0 gm of Bacto peptone; 5.0 gm of proteose peptone; 0.5 gm of ferric ammonium citrate; 1.0 gm dipotassium phosphate; 0.08 gm sodium thiosulphate and 15.0 gm of agar in 1.0 litre of distilled water.

Urea agar slant medium was prepared by dissolving 1.0 gm of peptone; 1.0 gm of dextrose; 5.0 gm of sodium chloride; 2.0 gm of dipotassium phosphate and 15.0 gm of agar in 1.0 litre of distilled water.

Koser's citrate medium was prepared by dissolving 1.5 gm of sodium ammonium phosphate; 1.0 gm of monobasic potassium phosphate anhydrous, 0.2 gm of magnesium sulphate anhydrous and 3.0 gm of sodium citrate in 1.0 litre of distilled water. The PH was adjusted at 6.8.

Litmus milk medium was prepared in 1.0 litre of fresh skim milk to which was added 1.0 ml of azolitmus indicator (0.5 gm dissolved in 10.0 ml of distilled water with a few drops of PH(NaOH).

Blood agar medium plates were prepared by dissolving 20.0 g of Tryptose; 5.0 g of sodium chloride and 15.0 g of agar in 1.0 litre of distilled water. The mixture was dissolved and homogenised by heating. Each plate also received 2.0 ml of 5.0 per cent of defibrinated sheep blood.

Semi-solid agar gelatin motility medium was prepared by dissolving 10.0 g of (Bacto) peptone; 3.0 g of meat extract (Bacto); 5.0 g of sodium chloride and 4.0 g of agar in 400 ml of distilled water (Solution A). The second solution (Solution B prepared by dissolving 80.0 g of gelatin in 600 ml of distilled water was mixed with solution A and homogenized by boiling. Each tube received 5.7 ml of the above mixture.

Carbohydrate medium was prepared by dissolving 10.0 g of peptone and 5.0 g of sodium chloride in 1.0 litre of distilled water. The pH was adjusted at 7.0. One hundred ml portions of the above broth were dispensed in five flasks. Maltose, sucrose, glucose, mannitol and lactose in 1.0 g quantities were individually added to each of the five flasks. A 1.0 ml portion of Andrade's indicator (containg 0.5 g of acid fuchsin in 100 ml of distilled water and 10.0 ml of 4.0 per cent sodium hydroxide was also added to each flask. The medium was autoclaved at 15.0 lbs pressure for 15 minutes.

PREPARATION OF STAINS.

Gram stain: A 95 per cent solution of crystal violet was made by dissolving 2.0 g of the dye in 20 ml of 95 per cent ethanol. Ammonium oxalate solution was prepared by dissolving 0.8 g quantity in 300 ml of distilled water. The two solutions were later mixed in the ration of 1 part of Crystal violet with 4 parts of ammonium oxalate (V/V).

Iodine solution: This was prepared by dissolving 1.0 g iodine crystals and 2.0 g of potassium iodide in 300 ml of distilled water.

Carbol fuchsin: This solution was prepared by mixing 5.0 g of fuchsin; 0.95 g of phenolcrystal and 50 ml of 95 per cent ethanol in 500 ml of distilled water.

Spore stain:

(a) Carbol fuchsin.

(b) 1 per cent hydrochloric acid.

(c) Methylene blue (95 per cent dye)-1.0 g dissolved in 95 per cent ethanol in 100 ml of distilled water. The two solutions (30 ml methylene blue and 100 ml of potassium hydroxide) were finally mixed together.

Capsule stain: was prepared by adding 5.0 g of Eirosine in 100 ml of distilled water. The mixture was boiled in water bath and 0.5 ml of 30 per cent formalin was subsequently

added as a preservative.

Flagellar stain (Lefson's) modification: The stain was prepared by the addition of tannic acid, 0.95 g; Sodium chloride; 0.50 g; Pararosaniline acetate 0.35 g and 35 ml of 95 per cent ethyl alcohol in 65 ml of distilled water. The above solution was found stable in about 10 days time. For Flagellar staining basic fuchsin solution was used as a counter stain.

PREPARATION OF REAGENTS:

Methyl red (MR) test indicator.

Methyl red (MR) test indicator (0.04 per cent) was prepared by dissolving 0.1 g of methyl red in 300 ml of 95 per cent ethyl alcohol.

Voges-Proskauer test Reagents:

The reagent was prepared by mixing the solution as per below:-

(a) 0.6 ml of 5 per cent alpha napthol in absolute ethyl alcohol.

(b) 0.2 ml of 40 per cent solution of sodium hydroxide mixed with the above.

Nitrate to Nitrite reduction test Reagent.

The reagents were made by the addition of the following:-

Solution A.

Sulfanilic acid (0.8 g) was dissolved in Acetic acid

5/V 100 ml (1 part of glacial acetic acid in 2.5 parts of distilled water (V/V).

Solution B.

Naphthylamine 0.5 g dissolved in 100 ml of 5/V glacial acetic acid.

Indol test Reagent.

The solution for this test was made by adding the following:-

Solution A.

It was prepared by dissolving 4.0 g of p-dimethylaminobenzaldehyde in mixture of ethanol (300 ml) and concentrated hydrochloric acid (80 ml).

Solution B.

This consisted of 1 per cent aqueus solution of potassium persulfate.

Urease test Reagent.

Solution A.

It was made by dissolving 2.0 g of urea in 20 ml of 95 per cent ethanol and 4.0 ml of distilled water.

Solution B.

Consisted of the following:-

Potassium hydrogen phosphate (K_2HPO_4), 0.1 g;

Potassium dihydrogen phosphate (KH_2PO_4), 0.1 g; Sodium chloride 5 g; 0.1 per cent phenol red; 1.0 ml and sterile distilled water 100 ml.

Solution A was diluted with Solution B in the ratio of 1:9 (V/V).

Carbohydrate fermentation test Reagent.

The indicator was prepared by adding 5.0 g of acid fuchsin to 100 ml of distilled water alongwith 16.0 ml of N/1 Sodium hydroxide.

Litmus milk test Reagent.

Indicator for this test was prepared by adding a few drops of N/N Sodium hydroxide to 5.0 g of azolitmus dissolved in 100 ml of distilled water.

MORPHOLOGICAL STUDIES.

All the slides for microscopic studies were prepared from broth culture grown for 24 hrs. Pure culture smears were appropriately stained for studying the morphology of the organisms.

Gram stain: The smears were stained in crystal violet for one minute and the excess stain was washed off in running tap water. Smears were then immersed in Gram's iodine solution for one minute and later washed in water. The

violet stain was decolourised with 95 per cent alcohol and the smears were subsequently stained with carbol fuchsin for 30 seconds. The slides were again washed in running tap water. The smears were dried and examined.

Spore stain: The smears were flooded with carbol fuchsin solution and kept in a water bath for 3 minutes at 37 C. The slides were cooled at room temperature and washed with tap water. The slides were passed through 95 per cent alcohol for 3 seconds and later washed in water. The slides were further stained with methylene blue solution for 1 minute and the excess stain was washed in water.

Capsule stain: The bacterial smears were made from organisms grown in 6 per cent dextrose solution on clean glass slides. A thin film of nigrosine solution was spared over the smear and slides were dried. The smears were subsequently stained with methylene blue for 3 minutes washed with water and dried.

BIOCHEMICAL TESTS.

Methyl red test was performed in nutrient broth culture tubes which were inoculated with test organisms and incubated at 37 C for 48 hrs. About five drops of 0.4 per cent solution of methyl red and 3.0 ml of n. 1 per cent solution of sodium hydroxide were also added to each culture tube. The development of pink colour was indicative of the positive test.

Vogues Praskeur (Active-methyl carbinol production) test.

Nutrient broth culture tubes were inoculated with each of the test organisms and were kept for incubation at 37 C for 48 hrs. 0.6 ml of 5 per cent alpha-napthol and 0.2 ml of 40 per cent sodium hydroxide were also added to each culture tube. The appearance of pink colour indicated the production of acetyl-methyl carbinol.

Reduction of nitrates to nitrite test.

The test organisms were inoculated in nitrite broth medium. At the end of 48 hrs incubation at 37 C, 1.0 ml of the test reagent prepared as described previously was added to each tube. The appearance of a distinct red colour was taken as an indication of a positive test.

Indol test.

Three nutrient broth culture tubes were inoculated for each test organism. After 48 hrs of incubation, 1 ml appropriate test reagents were added. The appearance of a red ring in the tubes indicated the production of indol from tryptophane.

Hydrogen sulphide test.

Nutrient broth culture tubes were inoculated with each of the test organisms. A strip of sterilized filter paper dipped in a 1 per cent lead acetate solution and later dried was introduced into each culture tube. Appropriate control tubes

were also similarly treated. All the tubes were incubated at 37° C for 48 hrs. The appearance of black precipitate on the paper strip indicated the production of hydrogen sulphide.

Urease test.

Urease production tests were performed on urea agar slants. The tubes for actual tests were inoculated along with the control tubes and incubated at 37° C for 48 hrs. Proper test reagents (0.1 ml) were then added to each tube. The urease activity was detected by the development of pink colour.

Koser citrate test.

The test was performed in citrate medium. The test organisms were inoculated in the medium and incubated at 37° C for 48 hrs. At the end of the incubation period, the utilization of citrate as the sole source of carbon was observed in all the culture tubes.

Carbohydrate fermentation test.

The test organisms were cultured in nutrient broth medium. The culture tubes also contained Durham tubes for detecting any gas production. All the test organisms alongwith appropriate controls were separately inoculated.

Litmus milk test.

Litmus milk test was performed in litmus milk medium which also contained azolitmus as an indicator. The culture tubes were incubated at 37 C for 48 hrs, for any gas production. All the test organisms alongwith appropriate controls were separately inoculated.

Action on blood.

Some blood agar plates were also inoculated with the three test organisms. Any change within the blood agar medium was read along with the uninoculated controls.

Estimation of bacterial growth.

Total counts

Total bacterial counts were made in Neubaner hemacytometer counting chamber. All counts were made in duplicate. Total number of organisms in all the four squares on the corners were counted for calculating the average. An average of two counts was taken for establishing the final number. The actual calculations were made as follows:-

Calculations:

Bacterial culture in 1.10 dilution.

N = the average number of cells counted in all the four squares (0.5 x 0.5 x 1).

Volume of each square will be 0.00025 cmm or 25×10^{-8} ml
since 1 cubic mm (cmm) = 10^{-3},
00025 cm = 25×10^{-8} ml.
therefore the number of bacteria per ml will be $\frac{N \times 10}{25 \times 10^{-8}}$

Turbidimetric method.

Growth estimations were also made by making use of the turbidimetric method. Growth estimations in different batches of bacterial cultures for experimental purposes were made by calculating the per cent transmittance (%T) and the optical density (OD) in a Bosch and Lomb spectronic 20 spectrophotometer at a wave length of 400 millimicrons.

Disc method.

Growth inhibitions in the various antibiotic sensitivity tests were also determined by measuring the zone of inhibition. The inhibition zones were measured on the millimeter scale and diameter of 15 mm and above was taken as an index of inhibition.

Antibiotic sensitivity tests.

The antibiotic sensitivity tests on the mycetomal bacteria were done by making use of a large number of broad spectrum antibiotics. The antibiotics were used in different concentrations and the inhibiting effects against each one of them was calculated either by the turbimetric method or by the disc method.

Turbidimetric method.

Sensitivity or resistance of the organisms against the various antibiotics was determined by broth dilution technique. Bacterial cultures were made in nutrient broth. The initial inoculum for antibiotic testing was kept uniform by making the total cell count in the hemacytometer counting chamber. Four different antibiotic concentrations in the range of 0.5 mg/ml to 2.0 mg/ml were prepared in distilled water. To each culture tube containing 10 ml of nutrient broth was added, 1.0 ml of a 24 hr old bacterial culture and 1.0 ml of each of the above antibiotic solution. The control tubes were similarly prepared, except that the antibiotic solution in each tube was replaced by an equal amount of normal saline. The experimental and the control tubes were incubated for 24 hrs at 37 C. The turbidity as a direct function of growth in every tube was measured in spectronic 20 by the previously described method. The concentrations of the various antibiotics which were used in the sensitivity tests ranged from 0.5 mg/ml to 2.0 mg/ml.

Disk method.

A confluent bacterial growth was first obtained on nutrient agar plates by inoculating the organisms and allowing a period of 8 hrs of incubation. Antibiotic soaked what man No.2 filter paper disc in various concentrations were first dried and placed over the bacterial carpet. The plates were incubated for a

period of 24 hrs at 37 C and the zones of inhibitions were measured in the manner described earlier. The concentrations of the various antibiotics used in the above experiments were as follows:-

1.	Achromycin	...	0.25mg/ml
2.	Chloromycetin	...	0.25 mg/ml.
3.	Streptomycin	...	1.0 mg/ml.
4.	Terramycin	...	0.25 mg/ml.
5.	Erythromycin	...	0.25 mg/ml.
6.	Furadantin	...	1.0 mg/ml.
7.	Penicillin	...	200 International Units (1 U)
8.	Sulphathiazol	...	1.0 mg/ml.

III- RESULTS

ISOLATION AND MORPHOLOGY

The isolated mycetomes from three different insects were transferred into tubes containing 15 ml sterile nutrient broth for making primary cultures. The tubes were incubated at 0,4, 25, 37 degree C and at room temperatures respectively. After 48 hours of incubation the bacterial columns were harvested by centrifugation at 1000 rpm and were resuspended in 0.85 per cent sterile normal saline. The above cell suspensions in 0.5 ml aliqnot were subsequently used for inoculating the various types of solid media. The bacterial growth on the variously used media presented the following characteristics:-

Nutrient agar: The colonies were raised, smooth, glistening and were having entire margins.

Potato agar: The colonies were raised, slimy and somewhat yellowish with entire margins.

Blood agar: The colonies were white, convex and had smooth surfaces.

Gelatin stab: Growth was dirty white, mostly on the surface with no liquification. Some gas bubbles were visible.

Nutrient broth: All tubes showed turbidity.

MICROSCOPIC EXAMINATION:

Microscopically, the organisms revealed the following morphological characteristics:-

Reation in Gram's staining	...	Grams negative.
Spore staining	...	Non-spore former.
Flagellar staining	...	Non-flagellated.
Capsule staining	...	Capsulated.
Mortility	...	Non-motile.

In general appearance, the organisms were short, somewhat oval bacilli having an average size of about 5.6 microns in length by 0.4 microns in width. The organisms were arranged in pairs.

BIOCHEMICAL TESTS.

The organisms gave a negative methyl red test (MR) and were found positive for Acetylmethyl cabinol (VP) production. The organisms reduced nitrate but were not able to produce Indol and hydrogen sulphide. They were able to utilize citrate and sugars. The organisms were able to ferment lactose and glucose with the production of acid and gas.

Sucrose, maltose and mannitol were fermented with the production of acid only. Litmus milk test was positive for acid with no coagulation. Table(I)shows the complete details of the various biochemical test. There was no haemolysis of

any kind of blood agar plates. On the basis of the above morpholo·ical and biochemical characterisation; all the bacter!al organisms which were isolated from the mycetomes of the three insects were identified as only one type of organisms belonging to Klebsiella spp.

ESTIMATION OF BACTERIAL GRO TH:

Total count:

All initial cultures were made in nutrient broth medium. A starting inoculum of 0.5 ml was used for inoculating the culture tubes. The tubes were incubated at 37 c for 24 hrs. Total cell counts were made in Neubauer haemacytometer counting chamber from a 24 hr. old culture whose turbidity was adjusted at 40 per cent transmittance in Spectronic 20 Bauch and Lomb spectrophotometer at a wave length of 400 millimicrons. An average of three hemacytometer counts was used to calculate the approximate number of bacteria. A 24 hrs culture at 40 per cent transmittance was found to contain 20 x 10^{-6} bacterial cells per ml.

Turbidimetric method:

Standardised bacterial culture tubes were first adjusted to a uniform population density at 40 per cent transmittance in the spectrophotometer. The antibiotic solutions were then added as described earlier and the tubes were incubated for a period of 24 hrs at 37 C. At the end of the incubation period the

inhibitory effect of the various antibiotics was determined by reading the difference in the per cent transmittance or optical density in each tube.

All readings were made in the spectrophotometer at a wave length of 400 millimicron. Table (11) indicates the results of antibiotic sensitivity as obtained by reading optical density in Spectronic 20. The data obtained from the above experiment is further illustrated by plotting the optical density verses antibiotic concentrations (Figures 1-4).

Disk method:

Antibiotic-impregnated disk were placed on blood agar plates which were previously seeded with Klebsiella organisms. The plates were incubated for a period of 24 hrs. At the end of the incubation period zones of inhibition were measured from edge to edge in millimeter (mm) scale. Table(111) indicates the details of inhibition zones as obtained against various antibiotics.

TABLE-I

DETAILS OF THE BIOCHEMICAL REACTIONS.

S. No.	Source of mycetomal bacteria	BIOCHEMICAL REACTIONS													
		MR	VP	Nitrate	Indole	H_2S	Urea	Citrate	Lactose	Glucose	Sucrose	Maltose	Mannitol	Litmus	Hemolysis on blood agar plates
A	Pyrilla perpusilla Walker	(-)	(+)	(+)	(-)	(-)	(-)	(+)	AG	AG	A	A	A	A	(-)
B	Idiocerus clypealis Leth	(-)	(+)	(+)	(-)	(-)	(-)	(+)	AG	AG	A	A	A	A	(-)
C	Ptyelus sexvittatus Walker	(-)	(+)	(+)	(-)	(-)	(-)	(+)	AG	AG	A	A	A	A	(-)

A = acid; AG = Acid and gas; H_2S = hydrogen sulphide; MR = methyl red; VP = acetyl methyl carbinol (-) = negative and (+) = positive

TABLE-II

OPTICAL DENSITY IN ANTIBIOTIC CONCENTRATIONS

S.No.	Source of mycetomal bacteria.	Panicillin mg/ml				Chloromycetin mg/ml				Streptomycin mg/ml				Erythromycin m/ml				Control
		0.5	1.0	1.5	2.0	0.5	1.0	1.5	2.0	0.5	1.0	1.5	2.0	0.5	1.0	1.5	2.0	
A	Pyrilla perpusilla Walker	.357	.347	.342	.337	.523	.469	.462	.456	.149	.137	.125	.114	.143	.131	.125	.122	.553
B	Idiocerus clypealis Leth	.284	.252	.248	.244	.292	.252	.248	.244	.456	.450	.297	.137	.137	.119	.114	.111	.469
C	Ptyelus sexvittatus Walker	.310	.292	.272	.252	.252	.222	.110	.01	.260	.244	.226	.028	.377	.337	.323	.310	.523

TABLE-III

ZONE OF INHIBITION IN ISOLATED MYCETOMAL BACTERIA

S.No.	Name of treatment	Concentration mg/ml	Inhibition zone (mm)	Remarks
1.	Achromycin	0.25	20	Inhibition
2.	Chloromycetin	0.25	22	Inhibition
3.	Streptomycin	1.0	30	Inhibition
4.	Terramycin	0.25	21	Inhibition
5.	Erythromycin	0.25	25	Inhibition
6.	Furadantin	1.0	20	Inhibition
7.	Pennicillin	200 IU	30	Inhibition
8.	Sulphathiazol	1.0	14	No Inhibition

Fig. 1. Shows plotting of optical density versus Penicillin concentrations as a measurement of growth inhibition.

SOURCE OF MYCETOMAL BACTERIA

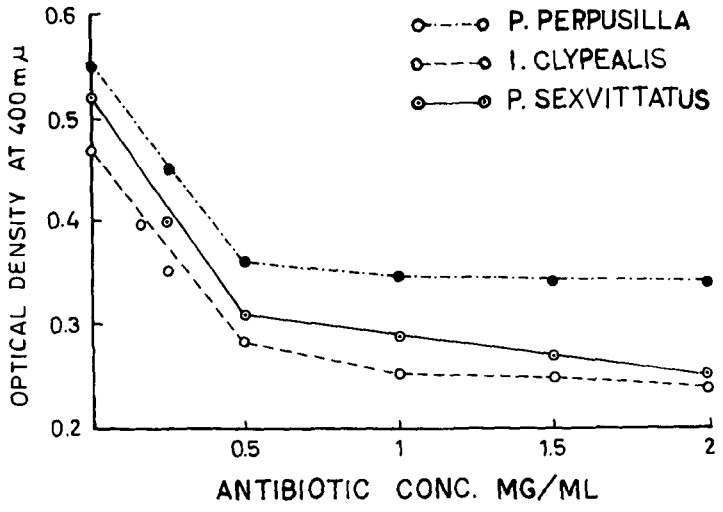


FIG.I

Fig.2. Shows plotting of optical density versus chloromycetin concentrations as a measurement of growth inhibition.

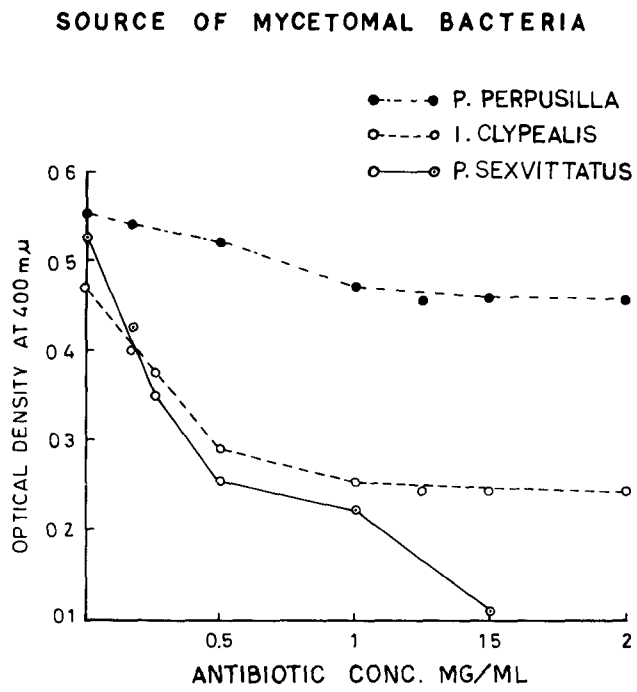

SOURCE OF MYCETOMAL BACTERIA
P. PERPUSILLA
I. CLYPEALIS
P. SEXVITTATUS
OPTICAL DENSITY AT 400 mμ
0.6
0.5
0.4
0.3
0.2
0.1
0.5
1
1.5
2
ANTIBIOTIC CONC. MG/ML

FIG.2

Fig.3. Shows plotting of optical density versus streptomycin concentrations as a measurement of growth inhibition.

SOURCE OF MYCETOMAL BACTERIA

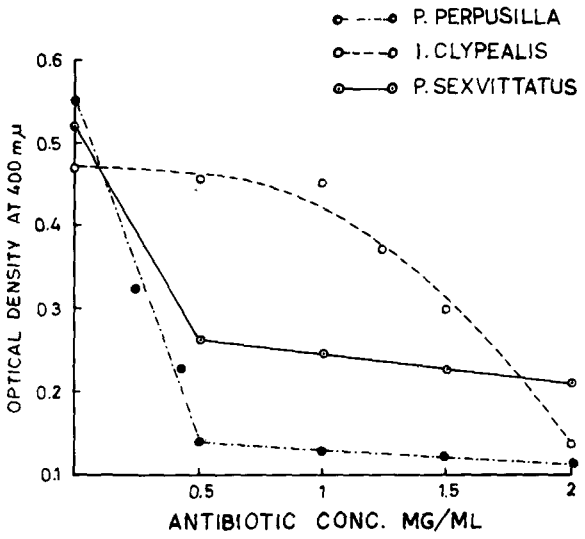


Fig.4. Shows plotting of optical density versus Erythromycin concentrations as a measurement of growth inhibition.

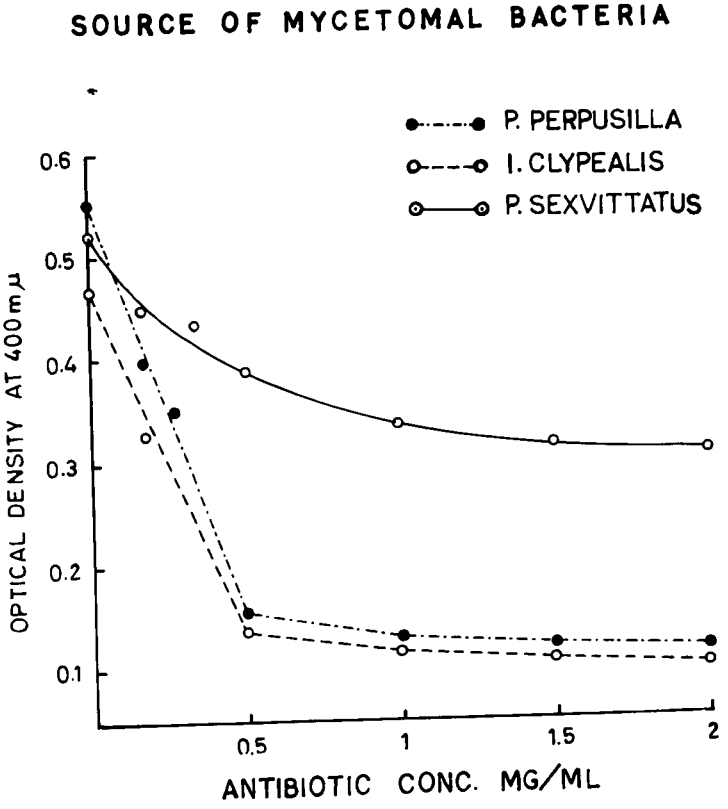

SOURCE OF MYCETOMAL BACTERIA
P. PERPUSILLA
I. CLYPEALIS
P. SEXVITTATUS
OPTICAL DENSITY AT 400 mμ
0.6
0.5
0.4
0.3
0.2
0.1
0.5
1
1.5
2
ANTIBIOTIC CONC. MG/ML

FIG.4

IV. DISCUSSION AND CONCLUSION

Although the microbiology of various orders of insects have been exhaustively studied by Glaser (1920), Gier (1946), Steinhaus (1949), Brooks and Richards (1955), Musgrave and Miller (1956), Brooks (1963), Le Blanc and Musgrave (1963), and Grinyer and Musgrave (1963), however there is a paucity of information regarding the microorganisms associated with Pyrilla perpusilla Walker, Idiocerus clypealis Leth and Ptyelus sexvittatus Walker. In the present investigations an attempt has been made to diagnose as accurately as possible within the limits of these studies, the mycetomal bacteria associated with the above insects. Morphological studies supplemented with the biochemical tests and growth of microorganisms have been used to diagnose the symbionts. Moreover, the effect of certain antibiotics and sulpha drugs have also been determined.

The bacteria constantly associated with the three insects were successfully raised on nutrient agar, potato agar, blood agar, gelatin agar and nutrient broth. They failed to liquefy gelatin and were gram-negative, non-sporer former, non-flagellated, non- motile and capsulated. They gave a positive acetyl-methyl-carbinol (VP) and negative methyl red(MR) tests. They reduced nitrates and were not able to produce indol and H2S. Citrate and other sugar were utilized profusely. Acid

and gas were produced when grown on lactose and glucose containing media, while in sucrose maltose and manitol containing media only acid was produced. Consequently the symbiotes in the mycetome of the three insects have been identified as Klebsiella species. These findings are, therefore, in accord with those of Steinhaus (1949), Steinhaus (1955) and Buchner and Stephens (1959) wherein they also found association of gram-negative bacteria with the mycetomes of coccids and grasshoppers respectively, and not that of Crawford et al (1960) who failed to isolate gram-negative bacteria with the mycetomes of weevil.

In-vitro studies dealing with antibiotic senstivity pattern showed that streptomycin and penicillin produced marked inhibition zone (30 mm) it was followed by erythromycin, chloromycetin, terramycin and achromycin. In test where inhibition was measured by using optical density as criterion of inhibition chloromycetin was most pronounced (Table II-III). Thus this finding in a way confirms the result of Frank (1954 and 1955) wherein he found the complete elimination of symbiotes when the host were fed upon penicillin and terramycin and also those of Brooks (1954), Brooks and Richards (1955), Hugger (1956), Schnieder (1956), Gabrani (1970) and Geigy et al (1954) and the two sulpha drugs used t inhibition occured in furadantin while sulphatiazole failed to inhibit the growth and hence they are in agreement with the

results obtained by Behrenz and Pechnau (1959) who found sulpha drugs in the form of sulphanilamide was effective in inhibiting the growth of symbiote of Anobium punctatum.

Constant association of Klebsiella species with the mycetomes of the plant-feeding insects species viz. P.perpusilla I. clypealis and P. sexvittatus leads one to conclude that Klebsiella is a common symbiote of the above insects. The question whether this symbiotic relationship is neutralistic or antagonistic or mutalistic need to be further investigated. In all probability it is mutualistic, because the association of the mycetomes in certain other insects have been reported to be of mutual advantage Pant and Fraenkel (1950), Fraenkel (1952), Goodchild (1955), Baines (1956) and Brooks (1963).

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P A R T - C

REFERENCES

*Aschner, M. 1932. Experimentelle Untersuchungen Über die Symbiose der Kleiderlaus. Naturwissenschaften- 20: 501-505.

_____ 1934. Studies on the Symbiosis of the body-louse V: Elimination of the Symbionts by centrifugalisation of the eggs. Parasitology- 26: 309-314.

Baines, S. 1956. The role of the symbiotic bacteria in the nutrition of Rhodnius prolixus (Hemiptera). J. Exptl. Biol. 33: 533-541.

Baker, J.R. 1946. The histochemical recognition of lipine. Quart. J. Micr. Sci. 87: 441-470.

_____ 1950. Cytological Technique. 3rd Ed. London (Methuen).

Balbiani, E.G. 1866. Sur la reproduction et l'embryogenie des pucerons. Comnt. Rend. Acad. Sci. Paris, 62: 1231.

_____ 1869-1871. Memoire sur la generation des aphides. I-V. Ann. Sci. Nat. Zool., II, 5, 161 (art.2); 1 (art.9); 15, 1(art.1); 1(art.4).

*Bauesch, M. 1956. Zyotologische Beobachtungen an den Mycetocyten von Periplaneta americana L. Naturwissenschaften- 43: 359-360.

*_____ 1958. Beitrage zur Zyotologie Und Embryologie einiger Insekten-Symbiosen. Z. Morphol. Oekol. Tiere, 47: 436-488.

---

* Reference not seen in original.

Behrenz, K. and Technau,G. 1959. Versuche zur Bekampfung von Anobium punctatum mit symbionticiden. Z. angew. Entomol. 44: 20-28.

Beier, M. 1933. Nachtrage and Berichtigungen zu den einzelnen Insecten-Ordungen. In Handbuch der zoologie (edited by T. Kruumbach). Walter dex Grunter Co., Berlin. (Beiers articles; 4 end half, Insecta 3, No.14, 2457)Symbiotes; (Mallophaga)., 2473(Lice),2475.

*Bewig, K. and Schwartz, M. 1956. Uber die physiologie der Symbiose bei einigen blutsaugenden Insekten. Arch. Mikrobiol.24:174-208.

Bhatnagar, P.D.S. and Musgrave, A.J. 1970. Cytochemistry, morphogenesis, and tentative identification of mycetomal microorganisms of Sitophilus granarius L.(Coleoptera). Canadan J. Microbiol.16:1357-1362.

Blewett, M., and Fraenkel, G. 1944. Intracellular symbiosis and vitamin requirements of two insects, Lasioderma sericorne and Sitodrepa Panicea. Proc. Roy. Soc. London, B 132:213-221.

*Blochmann, F. 1884. Uber die metamorphose der kerne in den ovarialeiern und uber den Beginn der blastodermbidung bei dep Ameisen. Verhandl. Naturrist. Med. ver. 3: 243-247.

*_____ 1887. Uber die Richtungskorper bei Insekteneiren. Gagenbaurs Morph. Jahrb. 12: 544.

*_____ 1888. Uber das regelmassige vorkommen von bacterien xxxx ahnlichen Gebilden in den Geweben und Eienn verschiedener Insekten. Z. Biol. 24: 1-16.

Bonhag, P.F. 1955. Histochemical studies of the ovarian nurse tissues and oocytes of the milkweed bug, Oncopeltus fasciatus (Dallas). 1. Cytology nucleic acids and carbohydrates. J. Morphol. 96: 381-439.

Bonhag, P.F., and Arnold, W.J. 1961. Histology, Histochemistry and tracheation of the ovariole Sheaths in the American Cockroach Periplaneta americana (L). J. Morph. 108: 107-129.

Brooks, M.A. 1963 b. The Microorganisms of healthy insects. Insect Pathology.(E.A. Steinhaus; ed) 1: 215-250 Acad.Press New York.

Brooks, M.A., and Richards, A.G. 1954. The necessity for intracellular bacterioids (Symbiotes) in growth and reproduction of cockroaches (Blattarie). Anat. Rec. 120: 742.

_____1956. Intracellular symbiosis in cockroaches. III Reinfection of aposymbiotic cockroaches with Symbiotes. J. Exptl. Zool. 132: 447-466.

Brues, C.T., and Glaser, R.W. 1921. A symbiotic fungus occuring in the fat body of Pulvinaria innumerabilis Rath. Biol. Bull. Marine Lab; 40: 299-324.

Bucher, G.E., and J.M. Stephens. 1958. Bacteria of grasshoppers of Western Canada: 1. The Enterobacteriaceae. J. Insect Pathol. 1: 356-373.

Buchner, P. 1912 . Studien an intrazellularen symbioten. 1 Die symbioten der Hemipteren. Arch. Protistenk. 26: 1-116.

_____1921. Tier und Pflanze in intracellularer symbiose Gebruder Borntraeger, Berlin, 462.

Buchner, P. 1923. Studies an intracellularen symbioten. IV. Die Bakterien symbiose der Bettwanze. Arch. Protistenk. 46:225.

_____ 1930. "Tier und Pflanze in Symbiose." Gebruder Borntraeger, Berlin, 900.

_____ 1953. Endosymbiose der Tiere mit Pflanzlichen Mikroorganismen, 771 p.p. Birkhauser, Basel.

* _____ 1955 a. Endosymbiosestudien an Schildlausen II. Stictococcus diversiseta Z. Morphol. Oekol. Tiere 43:397-424.

* _____ 1955 . Endosymbiosestudien an Schildlausen III. Macrocerococcus und Puto, Zwei primitive Pseudococcinen. Z. Morphol. Oekol. Tiere 43: 523-577.

_____ 1957. Endosymbiosestudien an Schildlausen. IV. Hippeococcus eine myrmekophile Pseudococcine. Z. Morphol Oekol. Tiere. 45: 379-410.

_____ 1966. "Endosymbiosis of animals with plant microorganism". Wiley (Interscience) New York, 901 p.p.

Bush, G.L., and Chapman, G.B. 1961. Electron microscopy of symbiotic bacteria in developing oocytes of the American cockroach, Periplaneta americana J. Bact. 81: 267.

Carter, W. 1935. The Symbionts of Pseudococcus brevipes (CKL). Ann. ent. Soc. Amer. 28: 60-64.

_____ 1936. The Symbionts of Pseudococcus brevipes (CKL) in relation to a phytotoxic secretion of the insect. Phytopathology, 26: 176-183.

Chang, T.P., and Musgrave, A.J. 1969. Histochemistry and ultrastructure of the mycetome and its 'symbiotes' in the Pear Psylla, Psylla Pyricola Foerster (Homoptera). Tissue and Cell 1: 597-606.

Cleveland, L.R. 1926. Symbiosis among animals with special reference to termites and their intestinal flagellates. Quart. Rev. Biol. 1: 51-60.

_____ 1959. Sex induced with ecdysone Proc. nat. Acad. Sci. Wash. 45: 267-276.

Crawford, R.F., L.A. McDermot., A.J. Musgrave 1960. Microbiol isolation from the granary weevil Sitophilus granarius (L). (Coleoptera: Curculionidae). Canad. Ent. 92: 577-581.

Davey, K.G., and Webster, G. . 1967. The structure and secretion of spermatheca of Rhodnius prolixus Stal: a histochemical study. Can. J. Zool. 46: 653-657.

de Bary, A. 1879. Die Erscheinung der Symbiose. Karl. J. Trubner. Strassburg.30.

* Ehrhardt, P. 1962. Untersuchungen uber die vom Stoffwechselphysiologie/Megura viciae Buckt., einer phloemsaugenden Aphide. Z. Vergleich. Physiol. 46: 160-211.

* _____ 1963. Untersuchungen uber Bau Und Funktion des Verdauungstraktes Von Megura Viciae Buckt., (Aphidae, Homoptera) Unter besonderer Berucksichtigung der Nahrungsaufnahme Und der Honigtauabgabe. Z. Morphol. Oekol. Tiere 62: 597-677.

Ekblom, R. 1931. Cytological and biochemical researches into the intracellular symbiosis in the intestinal cells of Rhagium inquisitor L. 11. Skand. Arch. Physiol 64: 279-298.

Fink, F. 1952. — Morphologische und Physiologische Untersuchungen an den intrazellularen Symbionten von Pseudococcus citri Risso. Z. Morphol. Oekol. Tiere. 41-78-147.

Florence, L. 1924. — An intracellular Symbiont of the dog louse. Am.J.Trop.Med.4: 397.

Fraenkel, G. 1952. — The role of symbionts as source of vitamins and growth factors for their insect host. Symposium Sur la "Symbiose Chez Les insects" (Amsterdam 1951). Tijdschr. Entomol. 95: 183-196.

Fraenkel, G., and Blewett, M. 1943. — Vitamnis of the B.group required by insects. Nature. 151: 703.

Frank, H. 1954. — Einwirkung verschiedener Antibiotica auf die symbionten der Kuchenschabe. Blatta orientalis L. Und die dadurch bedingten Veranderungen am Wirtstier., Verhandl. Deut. Zch. Zool. Ges. Tubingen. pp. 381-389.

_____ 1956. — Entfernung der intrazellularen symbionten der Kuchenschabe Periplaneta orientalis L durch Einwirkung verschiedener Antibiotica, Unter besonderer Berucksichtingung der veraunderungen am Wirtstier Und an den Bakterien. Z.Morphol.Oekol.Tiere 44:329-366.

Gabrani, W.D. 1970. — On the functions of intracellular symbiots of Sitophilus oryzae Linn. Separatum Experientia.27: 107.

Geigy, R., Halff, L.A., and Kocher, V. 1954. — L'acide folique comme element important dans La Symbiose intestinale de Triatoma infestans. Acta Trop. 11: 163-166.

Gier, H.T. 1936. — The morphology and behaviour of the intracellular bacterioids of roaches. Biol. Bull. 71: 433.

Gier, H.T. 1946. Intracellular bacteroids in the Cockroach. J. Bact. 53: 173.

Glaser, R.W. 1920. Biological studies on Intracellular bacteria. Biol.Bull. Marine Biol. Lab.39: 515.

_____ 1930. The Intracellular 'Symbionts and the 'Rickttsia' Arch. Pathol. 9: 71-96.

Godchild, A.J.P. 1955. The bacteria associated with Trixama Triatoma infestans and some other species of Reduviidae. Parasitology. 45: 441-449.

*Grabner, K.E. 1954. Vergleichend morphologische und physiologische studien an Anobiiden- und cerambycidensymbionten. Z. Morphol. Oekol. Tiere 41:471-528.

Gresson, R.R.R., and Theadgold, L.T. 1960. An Electron microscope study of Bacteria in the oocytes and follicle cells of Blatta orientalis. Quart. J. Micro. Sci. 101: 295.

Griyer, I., and Musgrave, A.J. 1966. Ultrastructure and peripheral membranes of the mycetomal microorganisms of Sitophilus granarius (L) Coleoptera. J. Cell. Sci. 1: 181-186.

*Gropengiesser, C. 1925. Untersuchungen uber die Symbiose der Blatiden mit niederen pflanzlichen organismen. Zentr. Bakteriol.Parisitenk.64:495-511.

Gumpert, J. and Schwartz, W. 1963. Untersuchungen uber die symbiose von Tieren mit Pilzen und Bakterien. X. Die Symbiose der Triatominen. 3. Pantothensaurelieferung als Funktion der Symbionten. Z. Allgem. Mikrobiol. 3: 1-14.

*Henneguy, L.T. 1904. Les insects (Morphologie), reproduction, (embryogenie) Lecons recueillies part A. Lacillon et. C. Poirault. Masson et. Cie. Paris 804.

Weitz, E. 1927. Uber intrazellulare symbiose beiholzfressenden Kaferlaven. Z. Morphol. Oekol Tiere. 7: 279.

Wertig, M. 1921. Attempts to cultivate the bacteroids of the Blattidae. Biol. Bull. 41: 181-197.

Winde, R. 1971. The fine structure of the mycetome symbionts of Brevicoryne brassicae, Myzus pusilal and Macrosiphum rosae J. Insect. Physiology. 17: 2035.

Hoover, S.C. 1945. Studies on the bacteroids of Cryptocercus punctulatus. J. Morph., 76: 213-225.

Huger, A. 1956. Experimentelle Untersuchungen uber die Kunstliche Symbiontenelimination bei vorratsschadingen: Rhisopertha dominica F. (Bostrychidae) und Oryzaephilus Surinamensis L. (Cucujidae). Z. Morphol. Oekol Tiere 44: 626-701.

Huxley, T.H. 1858. On the agamic reproduction and morphology of aphids Translinn. Soc. London, 22: 193.

Jurzitza, G. 1963. Die wirkung des sulfanilamids auf die symbionten einiger Anobiiden. Z. Entomol. 62: 302-306.

Keller, H. 1960. Die Kultur der intrazellularen Symbionten von Periplaneta Orientalis. Z. Naturforsch. 5b: 269-273.

Khan, Absar M. 1972. Observation on the mycetomes of Pyrilla perpusilla (Hemiptera: Lophopidae). Bull . . Ent. 1:54-60.

Koch, A. 1931 Die Symbiose von Oryzaephilus-Surinamensis L. Z. Morphol Oekol Tiere., 23: 389.

_____1936 Symbiosestudien II experimentalle Untersuchungen an Oryzaephilus Surinamensis L. Z. Morphol. Oekol Tiere., 32: 137-180.

Koch, A. 1955. Das verbaltnis zwischen Symbiont Und wirt. Verhandl. Deut. Zool. Ges in Erlange., 328.

_____ 1956 The experimental elimentation of symbionts and its consequences. Exp. Parasitology 5: 481-518.

_____ 1957. Die Physiologische Bedentung der symbionten Fur den. Wirtzorganismus. Ver Handl. devt. Ges. Inn. Med. 63, 55.

_____ 1959. Nevere und neveste unterschungen aus dem. 'Paul-Buchner Institute Fur Experimentelle Symbionses Forschuna symp genet. briol. Halatti del simposisa internazionale di Biologia experientale)9: 1.

_____ 1960. Intracellular symbiosis in insects. Ann. Rev. Microbiol. 14: 121-140.

Pohler, H., and Schwartz, M. 1962 a. Untersuchungen uber die symbiose von Tieren mit Pilzen und Bakterian VIII. Reinkultur und Identifizierung der Symbionten der coccidan Pseudococcus citri, P. maritimus and Orthezia insignis. Z. Allgem. Mikrobiol. 2: 10-31.

_____1962b. Untersunchungen Uber die Symbiose von Tieren mit pilzen und Bakterien IX. Uber die Beziehungen zwischen symbionten und wirtsorgansmus bei Pseudococcus citri, P. maritimus und Orthezia insignis. Z. Allgem. Microbiol. 2: 190-208.

Kolb, G. 1959. Untersuchungen uber die Kernverhaltnisse und morphologischen Eigenschaften symbiontischer Mikro-organismen bei verschiedenen Insekten. Z. Morphol. Oekol Tiere 48:1-71.

*Kotter, L. 1955. Bakteriologische und mikrochemische untersuchungen an der Magenscheibe von Pediculus vestimenti Burm. Arch. Mikrobiol. 23: 38-66.

*Krassilstschik, J.1889. Sur less bacteries biophytes. Ann. Inst. Pasteur 3: 465-472.

*_____ 1890. Ueber eine neue kategorie von Bacterien (Biophyten) die innern organisms leben Und ihm. Nutzen bringen Biol. Zentr., 10: 421-425.

Lamb, M.P. and Hinde,R. 1966. Structure and development of the mycetome in the cabbage aphid, Brevicoryne brassicae. J. Invert. Pathol. 9: 3-11.

Lanham, U.M. 1952. Observation on the supported intracellular microorganisms in aphids. Science, 116: 459-460.

Lanham, U.M. 1968. The Blockman bodies: heredity intracellular symbionts in insects. Biol. Rev. 43: 269-286.

Lowis, G. Moriber 1956. A cyto-chemical study of hemipteran Spermatogenessis. J. Morphol. 99: 271-327.

Louis, C. 1967. Cytologieet cyto-chimie du mycetome de Pseudococcus maritimus (Ehrhorn) (Homeptera Coceidae) c.r. hebd. Seanc Acad. Sci. Paris. 265: 437-440.

Le Blanc, M.M., and Musgrave, A.J. 1963. Some microbiological studies of two species of aphids, Aphis fabae scop and Macrosiphum pisi (Harris) (Hemiptera), Homoptera: Aphididae). Can.J. Microbiol. 9: 65-73.

*Leydig, F. 1860. Einige Bemerkungen Uber die Entwicklnng der Blattlause. Z. Wiss. Zool., 2, 62.

Lysenko, O., and Slama, K. 1959. The relation between oxygen consumption and bacterial infection in Saw-flies. J. insect Pathol. 15,134.

Mahdihassan, S. 1946. Bacterial origin of some insect pigments. Nature. 158: 58-59.

_____ 1947. Cicadella Viridis: its symbiotes and their functions. Curr. Sci. 16: 58-59.

_____ 1957. The role of symbiosis in the genus Coyiococus. Zeit. f. angew Entom. 33: 108.

*_____ 1957. The symboites of Jassid and of a coccid. Z. angew Entomologie Bd. 41,267.

Malke, H. 1964 a. Production of aposymbiotic cockroaches by means of lysozyme. Nature. 204: 1223-1224.

_____ 1964b. Wirkung von lysozym auf die symbionten der Blattiden. Z. Allgem. Mikrobiol. 4: 88-91.

Mansour, T. 1934. On the intracellular microorganisms some Bostry child beetles. Quart. J. Microscope Sci. 77:243.

Mazia, D., Brewer, P. and Alfert, M. 1953. The Cytochemical staining and measurement of protein with mercuric bromophenol blue. Biol1 Bull., 104: 57-67.

McManus, J.F.A. 1960. Staining method Histological and Histochemical. Paul B. Hoeber.

*Mercier, L. 1906. Les Corps bacteroides de la blatte Periplaneta orientalis: Bacillus-Cuenotin sp. Compt. Rend. Soc. Biol. 58: 682-684.

*_____1907. Recherches Sur Les bacterioides des Blattides. Arch. Protistenk 9: 346-358.

*Metchnikoff, E. 1886. Embryologische studien an insekten. Corl. Mistt. Z. Wiss Zool. 16:23.

Murray, F.A., and Tiegs, O.J. 1931. The metamorphosis of Calendra oryzae.
Quart. J. Micr. Sci. 77: 405-435.

Musgrave, A.J. 1964. Insect mycetomes.
Canad. Ent. 96: 377-389.

Musgrave, A.J., and Grinyer, I. 1968. Membranes associated with the disintegration of mycetomal microorganisms of Sitophilus zea-mis (Mots). (Coleoptera).
J. Cell. Sci. 3: 65-70.

Musgrave, A.J. Grinyer, I., and Homan, R. 1962. Some aspects of the fine structure of the mycetomes and mycetomal microorganisms in Sitophilus (Coleoptera; Curculionidae.)
Canad. J. Microbiol. 8: 747-751.

Musgrave, A.J., and Homan, R. 1962. Sitophilus sasakii (Tay). (Coleoptera: Curculionidae) in Canada: Anatomy and infectional microorganisms of Sitophilus weevils.
Canad. J. Microbiol. 7: 842-843.

Musgrave, A.J. and Miller, J.J. 1969. Studies of the association between strains and species in Sitophilus weevils and their mycetomal microorganisms.
Proc. 10th. Intern. Congr. Entomol. Montreal. 2: 313-317.

Musgrave, A.J., and Singh, J.B. 1966. Histochemical evidence of nuclear equavalents in mycetomal microorganisms in Sitophilus granarius (Linn).
J. Invert. Pathol. 7: 269-270.

Naidu, M.B. 1945 a. A special technique for the identification of the membracidae species by the intracellular microorganisms of their tumours.
Curr. Sci. 14: 210.

_____ 1945 b. Symbiosis in spittle insect Ptyelus nebulosus Fabr.
Curr. Sci. 14: 331.

*Paillot, A. 1933. L' infection ehezles insects. 6 Palissier Trevous 535.

Pant, N.C., and Raenkel, G. 1960. The function of the symbiotic yeasts of two insect species, Lasioderma sericorne F. and Stegobium paniceum L. Science 112: 498-500.

_____1954 a. Studies on the symbiotic yeasts of two insect species Lasioderma sericorne F., and Stegobium paniceum L. Biol. Bull. 107: 420-432.

_____1954 b. On the function of the intercellular symbionts of Oryzaephilus surinamensis L. (Cucujidae, Coleoptera). J. Zool. Soc. India. 6: 173.

Pearse, A.G.E. 1968 Histochemistry, theoritical and applied, 3rd edition, 1, London (Churchill). pp 759.

Pierantoni, V. 1909. L'-origine di-alcuni organi d', Icerya Purchasi e la. simbiosi ereditaria. Boll. Soc. Nat. Napoli 23: 147-160.

_____ 1910. Origine e. Structure del Corpo. Ovale, del Dacylopius citri e del Corpo verde de II, Aphis-brassicae- Boll. Soc. Nat. Napoli., 24: 1.

Pierre, L.L. 1962. Synthesis of ascorbic acid by the normal fat-body of the cockroach, Leucophaea maderae (F), and by its symbionts. Nature 193: 904-905.

_____ 1964. Uricase activity of isolated symbionts and the aposymbiotic fat body of a cockroach. Nature 201: 54-55.

Portier, P. 1918. Less symbiotes. Masson et Cie., Paris. 315 pp.

*Puchta, O. 1955. Experimentelle untersuchungen uber die Bedeutung der symbiose der Kleiderlaus Pediculus vestimenti Burm. Z. Naturwissenschaften. 41: 71-72.

*Rau, A. 1943. Symbiose und symbionten werb bei den Membraciden (Homoptera-cicadina) Z. Morphol. Oekol Tiere.,39:369-522.

Rehman, K.A. and Nath,R. 1940. Bionomics and control of the Indian sugar-cane leaf-hopper Pyrilla perpusilla Walk. Bull. Ent. Res. London 31: 179-190.

Richard, A.G. and Brooks, M.A. 1958. Internal symbiosis in insects. Ann. Rev. Ent., 3: 37-56.

Sizki, M.T.M. 1954. Desoxyribonucleic acid in the symbiotic microorganisms of the cockroach, Blattella germanica Science N.Y. 120: 35-36.

Schoel, J. 1934. Beitrage zur kenntnis der Aphidensymbiose. Botan Arch. 35: 152-196.

*Selmair, E. 1962. Beitrage zur wirkung wachstumsfordernder staffe auf die Entwicklung der Blattiden. Z. parasitenk. 21: 321-362.

Sheewsbury, J.E.D. and G.L. Barson. 1954a. The flora of the alimentary tract of the common earwig (Forficula auricularia) J. Path. Bact. 68:634-638.

_____ 1954b. The flora of the alimentary tract of the common crane fly (Tipula oleracea) J. Path. Bact. 68: 638-642.

Singh, B.B. and Musgrave, A.J. 1966. Some studies of the chromatin and cell walls of the mycetomal microorganisms of Sitophilus granarius (L). (Coleoptera). J. Cell. Sci. 1: 175-180.

Steinhaus, E.A. 1946. Insect microbiology comstock publishing Co., Ithaca New York. pp. 763.

Steinhaus, E.A. 1949. Principles of insect pathology. McGraw Hill Book Co., New York and London.

_____ 1955. Observation on the symbiotes of certain coccid Hilgradia, 24: 185.

_____ 1956. Insect microbiology. Hafner. New York.

*Sulc, K. 1910 a. 'Pseudovittellus' und ahnliche Gewebe der Homopteren Sind Wohnstatten symbiotischer, Saccharomyceten Sitzber Bohn, Ges Wiss. Math. Naturaliss. Klasse., 3: 1 Art. III.

*_____1910 b. Symbiotosche-saccharomyceten der echten cicaden (Cicadiadne). Sitzber Bohn Ges.Wiss Math. Natur-Wiss. Klasse., 3: 1 Art XIV.

Toth, L. 1952. The role of nitrogen active microorganisms in the nitrogen metabolism of insects. 'Symposium sur lax symbiose chez les insectes". Amsterdam. (Tijdschr. Entomol, 95: 43.59.

_____1959. Plant animal symbioses in 'Handbuch der pflanzenphysilogie' (W.Ruhland, ed). Vol. II pp. 639-654 Sprunger, Berlin.

Trager, W. 1953. Mitochondria or microorganisms? Science 116: 332.

Uichanco, L.B. 1924. Studies on the embryology and postnatal development of the aphididae with special reference to the history of the 'Symbiotic organ' or 'mycetome'. Philippine J. Sci. 24: 143-247.

Wigglesworth, V.B. 1936. Symbiotic bacteria in a blood-sucking insect, Rhodnius prolixus Stal (Hemiptera, Triatomidae) Parasitology. 28: 284-289.

Wigglesworth, V.B. 1952. Symbiosis in blood sucking insects. Taidschr Ent. 95a 63-69.

_____ 1953. Principles of insects physiology. Methyen and Co., London.5th edition.

_____ 1955. The breakdown of the thoracic gland in the insect Rhodnius prolixus. J. exp. Biol. 32: 485-491.

_____ 1959. "Physiologie der Insekten" Birkhauser, Basel.

* Wheeler, W.M. 1889. The embryology of Blatta-germanica and Doryphora decemineata. J. Morphol. 3: 291-386.

* Witlaczil 1882. Zur Anatomie der Aphiden. Arb. Zool. Inst. Wien., 4: 397-441.

(*) Original not seen.